

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of)	
Tanel TENSON et al.)	Group Art Unit: 1636
Application No.: 10/531,870)	Examiner: N. Vogel
Filed: April 19, 2005)	Confirmation No.: 5979
For: SELECTION SYSTEM CONTAINING)	
NON-ANTIBIOTIC RESISTANCE)	
SELECTION MARKER)	

DECLARATION PURSUANT TO 37 C.F.R. §§ 1.821-1.825

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Lisa E. Stahl, declare as follows:

That the content of the paper copy of the Sequence Listing filed concurrently herewith and the content of the computer readable copy of the Sequence Listing submitted herewith, in accordance with 37 C.F.R. § 1.821(c) and (e), respectively, are the same in compliance with § 1.821(f).

That the submission, filed in accordance with 37 C.F.R. § 1.821(g)[or (h)], herein does not include new matter [or go beyond the disclosure in the international application].

I hereby declare that all statements made herein of my own knowledge are true and that all statements were made on information and belief and are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of

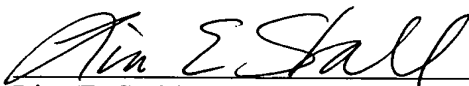
Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

BUCHANAN INGERSOLL & ROONEY PC

Date: September 4, 2007

By:

A handwritten signature in cursive script, appearing to read "Lisa E. Stahl", written over a horizontal line.

Lisa E. Stahl

Registration No. 56,704

P.O. Box 1404
Alexandria, VA 22313-1404
703 838 6609

L-ribulose-5-phosphate 4-epimerase protein sequences from different bacteria.

Blast query is L-ribulose-5-phosphate 4-epimerase protein sequence encoded by *E. coli araD* cds.

1.	<i>Shigella flexneri</i>	2
2.	<i>Salmonella typhimurium</i>	5
3.	<i>Salmonella enterica</i>	10
4.	<i>Yersinia pestis</i>	12
5.	<i>Pasteurella multocida</i>	14
6.	<i>Haemophilus influenzae</i>	16
7.	<i>Oceanobacillus ihayensis</i>	18
8.	<i>Bacillus halodurans</i>	20

1. Shigella flexneri

Length=231

Score = 474 bits (1220), Expect = 2e-132
Identities = 230/231 (99%), Positives = 231/231 (100%), Gaps = 0/231 (0%)

Query	1	MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVFVIKPSGVDYSVMTADDMVVVS	60
		ML+DLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVFVIKPSGVDYSVMTADDMVVVS	
Sbjct	1	MLKDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVFVIKPSGVDYSVMTADDMVVVS	60
Query	61	IETGEVVEGTTKKPSSDTPTHRLLYQAPPSIGGI VHTHSRHATIWAQAGQSIPATGTTHAD	120
		IETGEVVEGTTKKPSSDTPTHRLLYQAPPSIGGI VHTHSRHATIWAQAGQSIPATGTTHAD	
Sbjct	61	IETGEVVEGTTKKPSSDTPTHRLLYQAPPSIGGI VHTHSRHATIWAQAGQSIPATGTTHAD	120
Query	121	YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFPAWGKNA	180
		YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFPAWGKNA	
Sbjct	121	YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPFPAWGKNA	180
Query	181	EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ	231
		EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ	
Sbjct	181	EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ	231

LOCUS NP_706015 231 aa linear BCT 03-APR-2006
DEFINITION L-ribulose-5-phosphate 4-epimerase [Shigella flexneri 2a str. 301].
ACCESSION NP_706015
VERSION NP_706015.1 GI:24111505
DBSOURCE REFSEQ: accession NC_004337.1
KEYWORDS .
SOURCE Shigella flexneri 2a str. 301
ORGANISM Shigella flexneri 2a str. 301
Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
Enterobacteriaceae; Shigella.
REFERENCE 1 (residues 1 to 231)

AUTHORS Jin,Q., Yuan,Z., Xu,J., Wang,Y., Shen,Y., Lu,W., Wang,J., Liu,H., Yang,J., Yang,F., Zhang,X., Zhang,J., Yang,G., Wu,H., Qu,D., Dong,J., Sun,L., Xue,Y., Zhao,A., Gao,Y., Zhu,J., Kan,B., Ding,K., Chen,S., Cheng,H., Yao,Z., He,B., Chen,R., Ma,D., Qiang,B., Wen,Y., Hou,Y. and Yu,J.

TITLE Genome sequence of *Shigella flexneri* 2a: insights into pathogenicity through comparison with genomes of *Escherichia coli* K12 and O157

JOURNAL Nucleic Acids Res. 30 (20), 4432-4441 (2002)

PUBMED 12384590

REFERENCE 2 (residues 1 to 231)

CONSRTM NCBI Genome Project

TITLE Direct Submission

JOURNAL Submitted (18-OCT-2002) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA

REFERENCE 3 (residues 1 to 231)

AUTHORS Jin,Q., Shen,Y., Wang,J.H., Liu,H., Yang,J., Yang,F., Zhang,X.B., Zhang,J.Y., Yang,G.W., Wu,H.T., Dong,J., Sun,L.L., Xue,Y., Zhao,A.L., Gao,Y.S., Zhu,J.P., Chen,S.X., Yao,Z.J., Wang,Y., Lu,W.C., Qiang,B.Q., Wen,Y.M. and Hou,Y.D.

TITLE Direct Submission

JOURNAL Submitted (21-MAY-2001) State Key Laboratory for Molecular Virology and Genetic Engineering, Microbial Genome Center of Chinese Ministry of Public Health, 6 Rongjing Eastern Street, BDA, Beijing 100176, P.R.China

COMMENT VALIDATED REFSEQ: This record has undergone preliminary review of the sequence, but has not yet been subject to final review. The reference sequence was derived from AAN41722.
Method: conceptual translation.

LOCUS NP_835797 231 aa linear BCT 30-APR-2007

DEFINITION L-ribulose-5-phosphate 4-epimerase [*Shigella flexneri* 2a str. 2457T].

ACCESSION NP_835797

VERSION NP_835797.1 GI:30061626

DBSOURCE REFSEQ: accession NC 004741.1

KEYWORDS

SOURCE Shigella flexneri 2a str. 2457T

ORGANISM Shigella flexneri 2a str. 2457T

REFERENCE Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Shigella.

AUTHORS 1 (residues 1 to 231)

Wei, J., Goldberg, M.B., Burland, V., Venkatesan, M.M., Deng, W., Fournier, G., Mayhew, G.F., Plunkett, G. III, Rose, D.J., Darling, A., Mau, B., Perna, N.T., Payne, S.M., Runyen-Janecky, L.J., Zhou, S., Schwartz, D.C. and Blattner, F.R.

TITLE Complete Genome Sequence and Comparative Genomics of Shigella flexneri Serotype 2a Strain 2457T

JOURNAL Infect. Immun. 71 (5), 2775-2786 (2003)

PUBMED 12704152

REFERENCE 2 (residues 1 to 231)

CONSRM NCBI Genome Project

TITLE Direct Submission

JOURNAL Submitted (23-APR-2003) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA

REFERENCE 3 (residues 1 to 231)

AUTHORS Wei, J., Goldberg, M.B., Burland, V., Venkatesan, M.M., Deng, W., Fournier, G., Mayhew, G.F., Plunkett, G. III, Rose, D.J., Darling, A., Mau, B., Perna, N.T., Payne, S.M., Runyen-Janecky, L.J., Zhou, S., Schwartz, D.C. and Blattner, F.R.

TITLE Direct Submission

JOURNAL Submitted (13-JUN-2002) Genetics Laboratory, University of Wisconsin - Madison, 445 Henry Mall, Madison, WI 53706, USA

COMMENT VALIDATED REFSEQ: This record has undergone preliminary review of the sequence, but has not yet been subject to final review. The reference sequence was derived from AAP15602. Method: conceptual translation.

2. Salmonella typhimurium

Length=248

Score = 404 bits (1038), Expect = 2e-111
Identities = 196/208 (94%), Positives = 200/208 (96%), Gaps = 0/208 (0%)

Query	1	MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERG	VFKPSGVDYSVMTADDMVVVS	60
		MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERG	VFKPSGVDYSVMTADDMVVVS	
Sbjct	1	MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERG	VFKPSGVDYSVMTADDMVVVS	60
		MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERG	VFKPSGVDYSVMTADDMVVVS	
Query	61	IETGEVVEGTTKPPSSDTPTHRLLYQAPPSIGGIVH	THSRHATIWAQAGQSIPATGTTHAD	120
		+E+GEVVEG KKPSSDTPTHRLLYQAPP+IGGIVH	THSRHATIWAQAGQ IPATGTTHAD	
Sbjct	61	LESGEVVEGHKKPPSSDTPTHRLLYQAPPTIGGIV	THSRHATIWAQAGQPIPATGTTHAD	120
		LESGEVVEGHKKPPSSDTPTHRLLYQAPPTIGGIV	THSRHATIWAQAGQPIPATGTTHAD	
Query	121	YFYGTIPCTRKMWDAEINGEYEWETGNVIVETFEK	QGIDAAQMPGVLVHSHGPPFAWGKNA	180
		YFYGTIPCTRKMWT+AEINGEYEWETGNVIVETFEK	QGIDAAQMPGVLVHSHGPPFAWGKNA	
Sbjct	121	YFYGTIPCTRKMTEAEINGEYEWETGNVIVETFEK	QGIDAAQMPGVLVHSHGPPFAWGKNA	180
		YFYGTIPCTRKMTEAEINGEYEWETGNVIVETFEK	QGIDAAQMPGVLVHSHGPPFAWGKNA	
Query	181	EDAVHNAIVLEEVAYMGIFCRQLAPQLP	208	
		EDAVHNAIVLEEVAYMGIFCR L	P	
Sbjct	181	EDAVHNAIVLEEVAYMGIFCRHLRRSCP	208	

LOCUS	AAA27025	248 aa	linear	BCT 11-MAR-1994
DEFINITION	L-ribulose-5-phosphate 4-epimerase.			
ACCESSION	AAA27025			
VERSION	AAA27025.1	GI:153869		
DBSOURCE	locus STYARABAD accession M11047.1			
KEYWORDS	.			
SOURCE	Salmonella typhimurium			
ORGANISM	Salmonella typhimurium			
	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;			

Enterobacteriaceae; Salmonella.
 3 (residues 1 to 4790)
 Lin, H.C., Lei, S.P., Studnicka, G. and Wilcox, G.
 The araBAD operon of Salmonella typhimurium LT2. III. Nucleotide
 sequence of araD and its flanking regions, and primary structure of
 its product, L-ribulose-5-phosphate 4-epimerase
 Gene 34 (1), 129-134 (1985)
 3891514
 JOURNAL
 PUBMED
 COMMENT
 The sequence preceding araB coding region is part of the
 controlling region between the araC gene and araBAD operon. A
 potential ribosome binding site for the araB gene is located at
 positions 109-112. A 10-bp intercistronic region is located between
 the araB and araA genes. A potential ribosome binding site,
 'taagga', is located 7 bp distal from the start codon of araA. The
 site overlaps the stop codon of araB.
 A 143-bp intercistronic region exists between the araA and araD
 genes. The presumed ribosome binding site for araD is located at
 positions 3473-3475. This region contains several short
 complementary repeated sequences which can form stable stem-loop
 secondary structures. There is also a stem-loop structure 80 bp
 beyond the stop codon of araD which is followed by an A+T-rich
 sequence.
 Method: conceptual translation.

Length=231

Score = 462 bits (1189), Expect = 7e-129
 Identities = 223/231 (96%), Positives = 228/231 (98%), Gaps = 0/231 (0%)
 Query 1 MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVIKPSGVDYSVMTADDMVVVS 60
 MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVIKPSGVDYSVMTADDMVVVS
 Sbjct 1 MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVLVIKPSGVDYSVMTADDMVVVS 60
 Query 61 IETGEVVEGTTKPPSDTPTHRLLYQAFPSIGGIVHTHSRHATIWAQAGQSIPATGTTHAD 120

Sbjct 61 +E+GEVVEG KKPSSDTPTHRLLYQAFP+IGGIVHSHSRHATIWAQAGQ IPATGTTHAD 120
 LESEGEVVEGHKKPSSDTPTHRLLYQAFP+IGGIVHSHSRHATIWAQAGQIPATGTTHAD 120

Query 121 YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQIDAAQMPGVLVHSHGPFPAWGKNA 180
 YFYGTIPCTRKMT+AEINGEYEWETGNVIVETFEKQIDAAQMPGVLVHSHGPFPAWGKNA 180

Sbjct 121 YFYGTIPCTRKMTAEAEINGEYEWETGNVIVETFEKQIDAAQMPGVLVHSHGPFPAWGKNA 180
 YFYGTIPCTRKMTAEAEINGEYEWETGNVIVETFEKQIDAAQMPGVLVHSHGPFPAWGKNA 180

Query 181 EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ 231
 EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ 231

Sbjct 181 EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ 231
 EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ 231

LOCUS NP_459106 231 aa linear BCT 13-APR-2007
 DEFINITION L-Ribulose-5-phosphate 4-epimerase [Salmonella typhimurium LT2].

ACCESSION NP_459106

VERSION NP_459106.1 GI:16763491

DBSOURCE REFSEQ: accession NC_003197.1

KEYWORDS

SOURCE Salmonella typhimurium LT2

ORGANISM Salmonella typhimurium LT2

Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
 Enterobacteriaceae; Salmonella.

REFERENCE 1 (residues 1 to 231)

AUTHORS

McClelland, M., Sanderson, K.E., Spieth, J., Clifton, S.W.,
 Latreille, P., Courtney, L., Porwollik, S., Ali, J., Dante, M., Du, F.,
 Hou, S., Layman, D., Leonard, S., Nguyen, C., Scott, K., Holmes, A.,
 Grewal, N., Mulvaney, E., Ryan, E., Sun, H., Florea, L., Miller, W.,
 Stoneking, T., Nhan, M., Waterston, R. and Wilson, R.K.
 Complete genome sequence of Salmonella enterica serovar Typhimurium
 LT2

TITLE

JOURNAL

Nature 413 (6858), 852-856 (2001)

PUBMED 11677609

REFERENCE 2 (residues 1 to 231)

CONSRM NCBI Genome Project

TITLE Direct Submission

JOURNAL Submitted (10-SEP-2004) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA

REFERENCE 3 (residues 1 to 231)

CONSRTM NCBI Microbial Genomes Annotation Project

TITLE Direct Submission

JOURNAL Submitted (06-NOV-2001) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA

COMMENT VALIDATED REFSEQ: This record has undergone preliminary review of the sequence, but has not yet been subject to final review. The reference sequence was derived from AAL19065. Method: conceptual translation.

LOCUS AAL19065 231 aa linear BCT 09-AUG-2005

DEFINITION L-ribulose-5-phosphate 4-epimerase [Salmonella typhimurium LT2].

ACCESSION AAL19065

VERSION AAL19065.1 GI:16418599

DBSOURCE accession AE008698.1

KEYWORDS .

SOURCE Salmonella typhimurium LT2

ORGANISM Salmonella typhimurium LT2
Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Salmonella.

REFERENCE 1 (residues 1 to 231)

AUTHORS McClelland,M., Sanderson,K.E., Spieth,J., Clifton,S.W., Latreille,P., Courtney,L., Porwollik,S., Ali,J., Dante,M., Du,F., Hou,S., Layman,D., Leonard,S., Nguyen,C., Scott,K., Holmes,A., Grewal,N., Mulvaney,E., Ryan,E., Sun,H., Florea,L., Miller,W., Stoneking,T., Nhan,M., Waterston,R. and Wilson,R.K.

TITLE Complete genome sequence of Salmonella enterica serovar Typhimurium LT2

JOURNAL Nature 413 (6858), 852-856 (2001)

PUBMED 11677609

REFERENCE 2 (residues 1 to 231)

CONSRTM The Salmonella typhimurium Genome Sequencing Project

TITLE Direct Submission

JOURNAL Submitted (29-MAR-2001) Genome Sequencing Center, Department of

COMMENT

Genetics, Washington University School of Medicine, 4444 Forest
Park Boulevard, St. Louis, MO 63108, USA
Supported by NIH grant 5U 01 AI43283

Coding sequences below are predicted from manually evaluated
computer analysis, using similarity information and the programs;
GLIMMER; <http://www.tigr.org/softlab/glimmer/glimmer.html> and
GeneMark; <http://opal.biology.gatech.edu/GeneMark/>

EC numbers were kindly provided by Junko Yabuzaki and the Kyoto
Encyclopedia of Genes and Genomes; <http://www.genome.ad.jp/kegg/>,
and Pedro Romero and Peter Karp at EcoCyc;
<http://ecocyc.PangeaSystems.com/ecocyc/>

The analyses of ribosome binding sites and promoter binding sites
were kindly provided by Heladia Salgado, Julio Collado-Vides and
ReguonDB;
<http://kinich.cifn.unam.mx:8850/db/regulondb/intro.frameset>

This sequence was finished as follows unless otherwise noted: all
regions were double stranded, sequenced with an alternate
chemistries or covered by high quality data (i.e., phred quality >= 30); an attempt was made to resolve all sequencing problems, such as compressions and repeats; all regions were covered by sequence from more than one m13 subclone.

3. Salmonella enterica

Length=231

Score = 456 bits (1172), Expect = 6e-127
Identities = 220/231 (95%), Positives = 225/231 (97%), Gaps = 0/231 (0%)

Query	1	MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERG	VFIKPSGVDYSVMTADDMVVVS	60	
		MLEDLK QVLEANLALPKHNLVTLTWGNVSAVDRERG	VIKPSGVDYSVMTADDMVVVS		
Sbjct	1	MLEDLKHQVLEANLALPKHNLVTLTWGNVSAVDRERG	VLVIKPSGVDYSVMTADDMVVVS	60	
Query	61	IETGEVVEGTTKPPSSDTPTHRLLYQA	PPSIGGIVHTHSRHATIWAQAGQSI	PATGTTTHAD 120	
		+ETGEVVEG KKPSSDTPTHRLLYQA	FP+IG IVHTHSRHATIWAQAGQ	IPATGTTTHAD	
Sbjct	61	LETGEVVEGHKKPPSSDTPTHRLLYQA	PPTIGSIVHTHSRHATIWAQAGQ	PIPATGTTTHAD 120	
Query	121	YFYGTIPCTRKM	TDAEINGEYEWETGNVIVETFEKQ	GIDAAQMPGVLVHSHGPF	AWGKNA 180
		YFYGTIPCTRKM	T+AEINGEYEWETGNVIVE FEKQ	GI+AAQMPGVLVHSHGPF	AWGKNA
Sbjct	121	YFYGTIPCTRKM	TEAEINGEYEWETGNVIVEAFEKQ	GINAAQMPGVLVHSHGPF	AWGKNA 180
Query	181	EDAVHNAIVLEEVAYMGIFC	QLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ	231	
		EDAVHNAIVLEEVAYMGIFC	QLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ		
Sbjct	181	EDAVHNAIVLEEVAYMGIFC	QLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ	231	

LOCUS NP_454713 231 aa linear BCT 04-APR-2006
DEFINITION L-ribulose-5-phosphate 4-epimerase [Salmonella enterica subsp. enterica serovar Typhi str. CT18].

ACCESSION NP_454713
VERSION NP_454713.1 GI:16759096
DBSOURCE REFSEQ: accession NC_003198.1

KEYWORDS .
SOURCE Salmonella enterica subsp. enterica serovar Typhi str. CT18
ORGANISM Salmonella enterica subsp. enterica serovar Typhi str. CT18
Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
Enterobacteriaceae; Salmonella; Salmonella enterica subsp. enterica

REFERENCE	serovar Typhi.
AUTHORS	1 (residues 1 to 231) Parkhill,J., Dougan,G., James,K.D., Thomson,N.R., Pickard,D., Wain,J., Churcher,C., Mungall,K.L., Bentley,S.D., Holden,M.T.G., Sebaihia,M., Baker,S., Basham,D., Brooks,K., Chillingworth,T., Connerton,P., Cronin,A., Davis,P., Davies,R.M., Dowd,L., White,N., Farrar,J., Feltwell,T., Hamlin,N., Haque,A., Hien,T.T., Holroyd,S., Jagels,K., Krogh,A., Larsen,T.S., Leather,S., Moule,S., O'Gaora,P., Parry,C., Quail,M., Rutherford,K., Simmonds,M., Skelton,J., Stevens,K., Whitehead,S. and Barrell,B.G.
TITLE	Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18
JOURNAL	Nature 413 (6858), 848-852 (2001)
PUBMED	11677608
REFERENCE	2 (residues 1 to 231)
AUTHORS	Parkhill,J.
TITLE	Direct Submission
JOURNAL	Submitted (25-OCT-2001) Submitted on behalf of the Salmonella sequencing team, Sanger Centre, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, UK
REFERENCE	3 (residues 1 to 231)
CONSRM	NCBI Genome Project
TITLE	Direct Submission
JOURNAL	Submitted (07-OCT-2001) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA
COMMENT	VALIDATED <u>REFSEQ</u> : This record has undergone preliminary review of the sequence, but has not yet been subject to final review. The reference sequence was derived from <u>CAD01258</u> .

4. Yersinia pestis

Length=267

Score = 365 bits (937), Expect = 1e-99

Identities = 173/231 (74%), Positives = 197/231 (85%), Gaps = 0/231 (0%)

Query	1	MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDREGRGVFIKPSGVDYSVMTADDMVVVS	60
		ML +LK+QVL ANLALP+HNLVT TWGNVSA+DR++G+ VIKPSGV+Y+ MT DDMVVV	
Sbjct	37	MLNELKQOVLAAANLALPRHNLVFTTWGNVSAIDRQKGLLVIKPSGVEYASMTLDDMVVVE	96
Query	61	IETGEVVEGTTKKPSSDTPTHRLLYQAFPSIGGIVHTHSRHATIWAQAGQSIPATGTTTHAD	120
		+E+G VVEG+KKPSSDT THR+LY FP IGGIVHTHSRHATIWAQAG +PA GTTHAD	
Sbjct	97	LESGNVVEGSKKPSSDTPTHRVLNLPFPQIGGIVHTHSRHATIWAQAGLDLPAWGTTHAD	156
Query	121	YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPPFAWGKNA	180
		YFYG+IPCTR MT EI G YEWETGNVIV+TF ++GI +P VLV+SHGPPFAWG +A	
Sbjct	157	YFYGSIPCTRLMTHEEIAGRYEWETGNVIVDTFHERGITPDAPVAVLVNSHGPPFAWGSSA	216
Query	181	EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ	231
		E+AVHNA+VLEE+AYMGIF RQL PQL DMQ LLDKHYLRKHG AYYGQ	
Sbjct	217	ENAVHNAVLEELAYMGIFSRQLNPQLGDMQPQLLDKHYLRKHGKDYYGQ	267

LOCUS	NP_669385	267 aa	linear	BCT 26-JAN-2006
DEFINITION	L-ribulose-5-phosphate 4-epimerase [Yersinia pestis KIM].			
ACCESSION	NP_669385			
VERSION	NP_669385.1	GI:22125962		
DBSOURCE	REFSEQ: accession NC_004088.1			
KEYWORDS	.			
SOURCE	Yersinia pestis KIM			
ORGANISM	<u>Yersinia pestis KIM</u>			
	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Yersinia.			

REFERENCE	1 (residues 1 to 267)
AUTHORS	Deng, W., Burland, V., Plunkett, G. III, Boutin, A., Mayhew, G.F., Liss, P., Perna, N.T., Rose, D.J., Mau, B., Zhou, S., Schwartz, D.C., Fetherston, J.D., Lindler, L.E., Brubaker, R.R., Plana, G.V., Straley, S.C., McDonough, K.A., Nilles, M.L., Matson, J.S., Blattner, F.R. and Perry, R.D.
TITLE	Genome Sequence of <i>Yersinia pestis</i> KIM
JOURNAL	J. Bacteriol. 184 (16), 4601-4611 (2002)
PUBMED	12142430
REFERENCE	2 (residues 1 to 267)
CONSRM	NCBI Genome Project
TITLE	Direct Submission
JOURNAL	Submitted (10-SEP-2004) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA
REFERENCE	3 (residues 1 to 267)
AUTHORS	Deng, W., Burland, V., Plunkett, G. III, Boutin, A., Mayhew, G.F., Liss, P., Perna, N.T., Rose, D.J., Mau, B., Zhou, S., Schwartz, D.C., Fetherston, J.D., Lindler, L.E., Brubaker, R.R., Plana, G.V., Straley, S.C., McDonough, K.A., Nilles, M.L., Matson, J.S., Blattner, F.R. and Perry, R.D.
TITLE	Direct Submission
JOURNAL	Submitted (21-FEB-2002) Genetics, University of Wisconsin, 445 Henry Mall, Madison, WI 53706, USA
COMMENT	VALIDATED <u>REFSEQ</u> : This record has undergone preliminary review of the sequence, but has not yet been subject to final review. The reference sequence was derived from <u>AAM85636</u> . Method: conceptual translation.

5. Pasteurella multocida

Length=243

Score = 352 bits (904), Expect = 7e-96
Identities = 166/231 (71%), Positives = 190/231 (82%), Gaps = 0/231 (0%)

```
Query 1 MLEDLKRQVLEANLALPKHNLVLTWGNVSAVDRERGVIKPSGVDYSVMTADDMVVVS 60
      MLE+LK++V EANLALPK+ LVT TWGNVS +DRE+ + VIKPSGV+Y MT +DMVVV
Sbjct 13 MLEELKQKVFEANLALPKYKLVTTWGNVSGIDREKNLVVIKPSGVEYDTMTVEDMVVVD 72

Query 61 IETGEVVEGTTKKPSSDTPTHRLLYQAFFPSIGGIVHTHSRHATIWAQAQOSIPATGTTHAD 120
      + TG+VVEG KKPSSDT TH LY+ FPS+GGIVHTHSRHATIWAQAQ+ + A GTTHAD
Sbjct 73 LFTGQVVEGNKKPSSDTATHLELYRQFPFSLGGIVHTHSRHATIWAQAQEDLIAAGTTHAD 132

Query 121 YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQGIDAAQMPGVLVHSHGPPFAWGKNA 180
      YFYG+IPCTRKMT AEI GEYE ETG VIVETF +GID +P VLVHSHGPPFAWG +
Sbjct 133 YFYGSIPCTRKMTPAEIQGEYELETGKIVIVETFRVRGIDPKDVPAPVLVHSHGPPFAWGTD 192

Query 181 EDVHNNAIVLEEVAYMGIFCRQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ 231
      ++AVHNA+VLEE+ YM +F RQL P L MQQ LLDKHYLRKHG AYYGQ
Sbjct 193 DNAVHNAVLEEIGYMNLFQRQLRPNLASMQQELLDKHYLRKHGKNAYYGQ 243
```

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LOCUS      NP_246181                      243 aa                linear   BCT 03-DEC-2005
DEFINITION L-ribulose-5-phosphate 4-epimerase [Pasteurella multocida subsp.
            multocida str. Pm70].
ACCESSION  NP_246181
VERSION    NP_246181.1  GI:15603109
DBSOURCE   REFSEQ: accession NC 002663.1
KEYWORDS   .
SOURCE     Pasteurella multocida subsp. multocida str. Pm70
            Pasteurella multocida subsp. multocida str. Pm70
ORGANISM   Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales;
```

REFERENCE	Pasteurellaceae; Pasteurella.
AUTHORS	1 (residues 1 to 243) May, B.J., Zhang, Q., Li, L.L., Paustian, M.L., Whittam, T.S. and Kapur, V.
TITLE	Complete genomic sequence of Pasteurella multocida, Pm70
JOURNAL	Proc. Natl. Acad. Sci. U.S.A. 98 (6), 3460-3465 (2001)
PUBMED	<u>11248100</u>
REFERENCE	2 (residues 1 to 243)
CONSRTM	NCBI Genome Project
TITLE	Direct Submission
JOURNAL	Submitted (13-SEP-2001) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA
REFERENCE	3 (residues 1 to 243)
CONSRTM	NCBI Microbial Genomes Annotation Project
TITLE	Direct Submission
JOURNAL	Submitted (25-JUN-2001) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA
COMMENT	VALIDATED REFSEQ: This record has undergone preliminary review of the sequence, but has not yet been subject to final review. The reference sequence was derived from AAK03328. Method: conceptual translation supplied by author.

6. Haemophilus influenzae

```
Score = 348 bits (893), Expect = 1e-94
Identities = 167/231 (72%), Positives = 187/231 (80%), Gaps = 0/231 (0%)

Query 1 MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVIKPSGVDYSVMTADDMVVVS 60
      ML LK++V EANLALPKH+LVT TWGNVSA+DRE+ + VIKPSGVDY VMT +DMVVV
Sbjct 13 MLAQLKKEVFEANLALPKHHLVTF TWGNVSAIDREKNLVVIKPSGVDYDVTENDMVVVD 72

Query 61 IETGEVVEGTTKKPSSDTPTHRLLYQAFFPSIGGIVHTHSRHATIWAQAQSIPATGTTHAD 120
      + TG +VEG KKPSSDTPH LY+ FP IGGIVHTHSRHATIWAQAG I GTTH D
Sbjct 73 LFTGNIVEGNKKPSSDTPTHLELYRQPHIGGIVHTHSRHATIWAQAGLDIIEVGTTHGD 132

Query 121 YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQIDAAQMPGVLVHSHGPFAGKNA 180
      YFYGTIPCTR+MT EI G YE ETG VIVETF +GI+ +P VLVHSHGPFAGK+A
Sbjct 133 YFYGTIPCTRQMTTKEIKGNYELETGKIVIVETFLSRGIEPDNIPAVLVHSHGPFAGKDA 192

Query 181 EDVHNAIVLEEVAYMGIFCRQLAPQLPDMQQTLDDKHYLRKHGAKAYYGQ 231
      +AVHNA+VLEEVAYM +F +QL P L MQ+ LLDKHYLRKHG AYYGQ
Sbjct 193 NNAVHNAVLEEVAYMNLFSQQLNPVLSPMQKDLLDKHYLRKHGQNAYYGQ 243

LOCUS H64108 243 aa linear BCT 15-MAY-1998
DEFINITION L-ribulose-phosphate 4-epimerase (EC 5.1.3.4) - Haemophilus
influenzae (strain Rd KW20).
ACCESSION H64108
VERSION H64108 GI:1074955
DBSOURCE pir: locus H64108;

summary: #length 243 #molecular-weight 27241 #checksum 6284
;
genetic: #start_codon GTG
```

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;
; superfamily: L-ribulose-phosphate 4-epimerase
;
PIR dates: 18-Aug-1995 #sequence_revision 18-Aug-1995 #text_change
15-May-1998

KEYWORDS
SOURCE
ORGANISM
arabinose metabolism; isomerase; zinc.
Haemophilus influenzae
Haemophilus influenzae
Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales;
Pasteurellaceae; Haemophilus.
1 (residues 1 to 243)
REFERENCE
AUTHORS
Fleischmann,R.D., Adams,M.D., White,O., Clayton,R.A.,
Kirkness,E.F., Kerlavage,A.R., Bult,C.J., Tomb,J.F.,
Dougherty,B.A., Merrick,J.M., McKenney,K., Sutton,G., FitzHugh,W.,
Fields,C., Gocayne,J.D., Scott,J., Shirley,R., Liu,L.I., Glodek,A.,
Kelley,J.M., Weidman,J.F., Phillips,C.A., Spriggs,T., Hedblom,E.,
Cotton,M.D., Utterback,T.R., Hanna,M.C., Nguyen,D.T., Saudek,D.M.,
Brandon,R.C., Fine,L.D., Fritchman,J.L., Fuhrmann,J.L.,
Georgiades,N.S.M., Gnehm,C.L., McDonald,L.A., Small,K.V.,
Fraser,C.M., Smith,H.O. and Venter,J.C.
TITLE
Whole-genome random sequencing and assembly of Haemophilus
influenzae Rd
JOURNAL
Science 269 (5223), 496-512 (1995)

```

7. Oceanobacillus iheyensis

Length=231

Score = 298 bits (763), Expect = 2e-79
Identities = 142/231 (61%), Positives = 170/231 (73%), Gaps = 1/231 (0%)

Query	1	MLEDLKRQVLEANLALPKHNLVLTWGNVSAVDRERG	VFKPSGVDYSVMTADDMVVVS	60
		MLE LK +V EANL LPK LV TWGN SA DRE G+FVKPSGVDY	M A DMVVV	
Sbjct	1	MLEQLKEEVFEANLIDLKQGLVKYTWGNASAFDRESGLF	VFKPSGVDYKTMKASDMVVVD	60
Query	61	IETGEVVEGTTKPPSSDTPTHRLLYQA	PPSIGGIVHTHSRHATIWAQAGQSIPATGTTHAD	120
		++ G VVEG PSSDT TH +LY+ +P +GGIVHTHS	AT+WAQAG +P GTTHAD	
Sbjct	61	LD-GNVVEGELNPSSDTATHAVLYKRYPELGGIVHTHS	TWATVWAQAGLDVPMGTTHAD	119
Query	121	YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQ	GIDAAQMPGVLVHSHGPPFAWGKNA	180
		FYG +PCTR +T EI+ YE ETG VI+ETFE++G+D	+PGVL+H H PF WGK+	
Sbjct	120	TFYGAVPCTRFLTQEEIDRGYEAGTGRVLIETFEER	GLDVFAIPGVLHGHAPFTWGKDV	179
Query	181	EDAVHNAIVLEEVAYMGIFCRQLAPQLPDMQOTLLD	KHYLRKHGAKAYYGQ	231
		+ AV N++VLEEVA M +F R+L P++ +LDKHYLRKHG	AYYGQ	
Sbjct	180	QSAVVNSVLEEVAKMNLFAELNRFAPELPDRILD	KHYLRKHGGDAYYGQ	230

LOCUS NP_693720 231 aa linear BCT 02-DEC-2005
DEFINITION L-ribulose-5-phosphate 4-epimerase [Oceanobacillus iheyensis HTE831].
ACCESSION NP_693720
VERSION NP_693720.1 GI:23100253
DBSOURCE REFSEQ: accession NC_004193.1
KEYWORDS .
SOURCE Oceanobacillus iheyensis HTE831
ORGANISM Oceanobacillus iheyensis HTE831
Bacteria; Firmicutes; Bacillales; Bacillaceae; Oceanobacillus.
REFERENCE 1 (residues 1 to 231)

AUTHORS	Takami, H., Takaki, Y. and Uchiyama, I.
TITLE	Genome sequence of <i>Oceanobacillus iheyensis</i> isolated from the Iheya Ridge and its unexpected adaptive capabilities to extreme environments
JOURNAL	Nucleic Acids Res. 30 (18), 3927-3935 (2002)
PUBMED	<u>12235376</u>
REFERENCE	2
AUTHORS	Lu, J., Nogi, Y. and Takami, H.
TITLE	<i>Oceanobacillus iheyensis</i> gen. nov., sp. nov., a deep-sea extremely halotolerant and alkaliphilic species isolated from a depth of 1050 m on the Iheya Ridge
JOURNAL	FEMS Microbiol. Lett. 205 (2), 291-297 (2001)
PUBMED	<u>11750818</u>
REFERENCE	3 (residues 1 to 231)
CONSRM	NCBI Genome Project
TITLE	Direct Submission
JOURNAL	Submitted (10-SEP-2004) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA
REFERENCE	4 (residues 1 to 231)
AUTHORS	NCBI Microbial Genomes Annotation Project.
TITLE	Direct Submission
JOURNAL	Submitted (24-FEB-2003) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA
COMMENT	VALIDATED <u>REFSEQ</u> : This record has undergone preliminary review of the sequence, but has not yet been subject to final review. The reference sequence was derived from <u>BAC14754</u> . Method: conceptual translation.

8. Bacillus halodurans

Length=231

Score = 293 bits (750), Expect = 5e-78

Identities = 146/231 (63%), Positives = 172/231 (74%), Gaps = 3/231 (1%)

```
Query 1 MLEDLRQVLEANLALPKHNLVLTWGNVSAVDRERGVIKPSGVDYSVMTADDMVVVS 60
      MLE LK V +ANL LPK+ LVT TWGNVS +DRE+G+ VIKPSGV+Y M + DMVVV
Sbjct 1 MLEQLKETVFKANLYLPKYQLVFTWGNVSGIDREKGLVVIKPSGVYEFEMKSKDMVVVD 60

Query 61 IETGEVVEGTTKKPSSDTPTHRLLYQAFFPSIGGIVHTHSRHATIWAQAGQSIPATGTTTHAD 120
      +E G +VEG KPSSDTPTH LY+AF +GGIVHTHS AT WAQAG+ IPA GTTHAD
Sbjct 61 LE-GNIVEGDLKPSSDTPTHLALYRAFDPKVGIVHTHSVWATAWAQAGKEIPAYGTTTHAD 119

Query 121 YFYGTIPCTRKMTDAEINGEYEWETGNVIVETFEKQIDAAQMPGVLVHSHGPFPAWGKNA 180
      YF+GTIPCTR MT+ EI G+YE ETGNVIVETF + D +PGVLVHSH PF WGK+
Sbjct 120 YFHGTIPCTRPMTETEILGDYEXETGNVIVETFRNK--DPMSIPGVLVHSHAPFVWGKDP 177

Query 181 EDVHNAIVLEEVAYMGIFCRQLAPQLPDMQOTLLDKHYLRKHGAKAYYGQ 231
      +AVH+A+VLEEVA M ++ + M LLD+H+ RKHG AYYGQ
Sbjct 178 MEAVHHAIVLEEVAKMAQKTLISERTLPMDSVLLDRHFYRKHGQAAYYGQ 228
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LOCUS      NP_242737                231 aa                linear    BCT 03-DEC-2005
DEFINITION L-ribulose-5-phosphate 4-epimerase [Bacillus halodurans C-125].
ACCESSION  NP_242737
VERSION    NP_242737.1  GI:15614434
DESOURCE   REFSEQ: accession NC 002570.2
KEYWORDS   .
SOURCE      Bacillus halodurans C-125
            Bacillus halodurans C-125
ORGANISM    Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
```

- REFERENCE 1 (residues 1 to 231)
AUTHORS Takami,H., Nakasone,K., Takaki,Y., Maeno,G., Sasaki,R., Masui,N., Fuji,F., Hirama,C., Nakamura,Y., Ogasawara,N., Kuhara,S. and Horikoshi,K.
TITLE Complete genome sequence of the alkaliphilic bacterium Bacillus halodurans and genomic sequence comparison with Bacillus subtilis
JOURNAL Nucleic Acids Res. 28 (21), 4317-4331 (2000)
PUBMED 11058132
- REFERENCE 2 (residues 1 to 231)
AUTHORS Nakasone,K., Masui,N., Takaki,Y., Sasaki,R., Maeno,G., Sakiyama,T., Hirama,C., Fuji,F. and Takami,H.
TITLE Characterization and comparative study of the rrn operons of alkaliphilic Bacillus halodurans C-125
JOURNAL Extremophiles 4 (4), 209-214 (2000)
PUBMED 10972189
- REFERENCE 3 (sites)
AUTHORS Takami,H. and Horikoshi,K.
TITLE Analysis of the genome of an alkaliphilic Bacillus strain from an industrial point of view
JOURNAL Extremophiles 4 (2), 99-108 (2000)
PUBMED 10805564
- REFERENCE 4 (sites)
AUTHORS Takami,H., Takaki,Y., Nakasone,K., Sakiyama,T., Maeno,G., Sasaki,R., Hirama,C., Fuji,F. and Masui,N.
TITLE Genetic analysis of the chromosome of alkaliphilic Bacillus halodurans C-125
JOURNAL Extremophiles 3 (3), 227-233 (1999)
PUBMED 10484179
- REFERENCE 5 (sites)
AUTHORS Takami,H., Masui,N., Nakasone,K. and Horikoshi,K.
TITLE Replication origin region of the chromosome of alkaliphilic Bacillus halodurans C-125
JOURNAL Biosci. Biotechnol. Biochem. 63 (6), 1134-1137 (1999)
PUBMED 10427704
- REFERENCE 6 (sites)
AUTHORS Takami,H., Takaki,Y., Nakasone,K., Hirama,C., Inoue,A. and

Horikoshi, K.
 Sequence analysis of a 32-kb region including the major ribosomal
 protein gene clusters from alkaliphilic *Bacillus* sp. strain C-125
 Biosci. Biotechnol. Biochem. 63 (2), 452-455 (1999)
 10192928
 7 (sites)
 Takami, H., Nakasone, K., Ogasawara, N., Hiram, C., Nakamura, Y.,
 Masui, N., Fuji, F., Takaki, Y., Inoue, A. and Horikoshi, K.
 Sequencing of three lambda clones from the genome of alkaliphilic
Bacillus sp. strain C-125
 Extremophiles 3 (1), 29-34 (1999)
 10086842
 8 (sites)
 Takami, H., Nakasone, K., Hiram, C., Takaki, Y., Masui, N., Fuji, F.,
 Nakamura, Y. and Inoue, A.
 An improved physical and genetic map of the genome of alkaliphilic
Bacillus sp. C-125
 Extremophiles 3 (1), 21-28 (1999)
 10086841
 9 (sites)
 Takami, H.
 Genome analysis of facultatively alkaliphilic *Bacillus* halodurans
 C-125
 (in) Extremophiles in deep-sea environments (Ed.);
 . HORIKOSHI, K. TSUJII;
 : 249-284; Springer-Verlag (1999)
 10 (sites)
 Takami, H. and Horikoshi, K.
 Reidentification of facultatively alkaliphilic *Bacillus* sp. C-125
 to *Bacillus* halodurans
 Biosci. Biotechnol. Biochem. 63, 943-945 (1999)
 11 (residues 1 to 231)
 NCBI Genome Project
 Direct Submission
 Submitted (13-SEP-2001) National Center for Biotechnology
 Information, NIH, Bethesda, MD 20894, USA

TITLE
 JOURNAL
 PUBMED
 REFERENCE
 AUTHORS

TITLE
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 PUBMED
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 PUBMED
 REFERENCE
 AUTHORS
 TITLE

JOURNAL
 (in) Extremophiles in deep-sea environments (Ed.);
 . HORIKOSHI, K. TSUJII;
 : 249-284; Springer-Verlag (1999)
 10 (sites)
 Takami, H. and Horikoshi, K.
 Reidentification of facultatively alkaliphilic *Bacillus* sp. C-125
 to *Bacillus* halodurans
 Biosci. Biotechnol. Biochem. 63, 943-945 (1999)
 11 (residues 1 to 231)
 NCBI Genome Project
 Direct Submission
 Submitted (13-SEP-2001) National Center for Biotechnology
 Information, NIH, Bethesda, MD 20894, USA

REFERENCE
 AUTHORS
 TITLE
 JOURNAL
 REFERENCE
 CONSRTM
 TITLE
 JOURNAL

REFERENCE	12 (residues 1 to 231)
CONSRM	NCBI Microbial Genomes Annotation Project
TITLE	Direct Submission
JOURNAL	Submitted (25-JUN-2001) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA
REFERENCE	13 (residues 1 to 231)
AUTHORS	Takami, H. and Takaki, Y.
TITLE	Direct Submission
JOURNAL	Submitted (22-MAR-2000) Japan Marine Science and Technology Center, Deep-sea Microorganisms Research Group, 2-15 Natsushima, Yokosuka, Kanagawa 237-0061, Japan
COMMENT	VALIDATED REFSEQ: This record has undergone preliminary review of the sequence, but has not yet been subject to final review. The reference sequence was derived from <u>BAB05590</u> .

The *Bacillus subtilis* L-arabinose (*ara*) operon: nucleotide sequence, genetic organization and expression

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and Hermínia de Lencastre^{1,2}

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Oeiras Codex, Portugal

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Microbiology, 1230 York
Avenue, New York, NY
10021-6399, USA

The *Bacillus subtilis* L-arabinose metabolic genes *araA*, *araB* and *araD*, encoding L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase, respectively, have been cloned previously and the products of *araB* and *araD* were shown to be functionally homologous to their *Escherichia coli* counterparts by complementation experiments. Here we report that *araA*, *araB* and *araD*, whose inactivation leads to an Ara⁻ phenotype, are the first three ORFs of a nine cistron transcriptional unit with a total length of 11 kb. This operon, called *ara*, is located at about 256° on the *B. subtilis* genetic map and contains six new genes named *araL*, *araM*, *araN*, *araP*, *araQ* and *abfA*. Expression of the *ara* operon is directed by a strong σ^A -like promoter identified within a 150 bp DNA fragment upstream from the translation start site of *araA*. Analysis of the sequence of the *ara* operon showed that the putative products of *araN*, *araP* and *araQ* are homologous to bacterial components of binding-protein-dependent transport systems and *abfA* most probably encodes an α -L-arabinofuranosidase. The functions of *araL* and *araM* are unknown. An *in vitro*-constructed insertion-deletion mutation in the region downstream from *araD* allowed us to demonstrate that *araL*, *araM*, *araN*, *araP*, *araQ* and *abfA* are not essential for L-arabinose utilization. Studies with strains bearing transcriptional fusions of the operon to the *E. coli lacZ* gene revealed that expression from the *ara* promoter is induced by L-arabinose and repressed by glucose.

Keywords: *Bacillus subtilis*, L-arabinose (*ara*) operon, expression, catabolite repression

INTRODUCTION

Bacillus subtilis, an endospore-forming Gram-positive bacterium, is able to grow on L-arabinose as sole carbon source. L-Arabinose residues are found widely distributed among many heteropolysaccharides of different plant tissues, such as arabinans, arabinogalactans, xylans and arabinoxylans. *Bacillus* species in their

natural reservoir, the soil, participate in the early stages of plant material decomposition and *B. subtilis* secretes three enzymes, an endo-arabanase and two arabinosidases, capable of releasing arabinosyl oligomers and L-arabinose from plant cell walls (Kaji & Saheki, 1975; Weinstein & Albersheim, 1979). The pathway of L-arabinose utilization in *B. subtilis* has been described by Lepesant & Dedonder (1967a). After entering the cell, L-arabinose is sequentially converted to L-ribulose, L-ribulose 5-phosphate, and D-xylulose 5-phosphate by the action of L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase, respectively. D-Xylulose 5-phosphate is further catabolized through the pentose phosphate pathway. Mutants unable to use L-arabinose as sole carbon source, deficient in one of the three enzymes involved in L-arabinose catabolism, have been characterized, as well as constitutive mutants for

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[‡] Present address: Public Health Research Institute, 455 First Avenue, New York, NY 10016, USA.

Abbreviations: Cm, chloramphenicol; Em, erythromycin; Km, kanamycin; Sp, spectinomycin.

The accession numbers for the nucleotide sequences reported in this paper are X89408 (*araA*, *B* and *D*) and X89810 (*araL*, *M*, *N*, *P*, *Q* and *abfA*).

all three enzymes (Lepesant & Dedonder, 1967a, b). The synthesis of these enzymes was shown to be inducible by L-arabinose and the isomerase activity is subjected to catabolite repression by glucose and glycerol (Lepesant & Dedonder, 1967a).

A collection of Ara⁻ *B. subtilis* mutants was isolated, biochemically characterized and the three metabolic genes, *araA*, *araB* and *araD*, encoding L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase, respectively, were identified and mapped between *aroG* and *leuA*, at about 256° on the *B. subtilis* genetic map (Paveia & Archer, 1992a, b). Two additional classes of mutations affecting L-arabinose utilization were identified; one included mutations conferring an Ara⁻ phenotype to strains bearing the *araA*, *araB* and *araD* wild-type alleles (Paveia & Archer, 1992a, b), and another comprised mutants showing constitutive expression of the three genes (Sá-Nogueira *et al.*, 1988). These mutations were mapped between the *cysB* and *hisA* markers, at about 294° on the *B. subtilis* genetic map, and define another *ara* locus named *araC*. Expression of L-arabinose isomerase is severely repressed during growth in media containing L-arabinose plus glucose. Since L-arabinose isomerase expression is still regulated by catabolite repression in strains which contain constitutive mutations (*araC^c*), L-arabinose transport does not play a major role in catabolite repression of expression of the metabolic enzymes (Sá-Nogueira *et al.*, 1988). The products of the previously cloned genes *araA*, *araB* and *araD* were shown in complementation experiments to be functionally homologous to their *Escherichia coli* counterparts. Transformation experiments involving defined restriction fragments from the cloned genes showed that they are adjacent and probably constitute an operon with the order *araABD* (Sá-Nogueira & Lencastre, 1989), unlike the *araBAD* order found in the *E. coli* operon (Englesberg *et al.*, 1969).

In this communication we report the cloning of an additional 7.1 kb chromosomal fragment, located downstream from *araD* and the nucleotide sequence of over 11 kb. This region contains a cluster of nine genes: the metabolic genes *araA*, *araB* and *araD*, and six new genes named *araL*, *araM*, *araN*, *araP*, *araQ* and *abfA*. We have demonstrated that all genes comprise a single transcriptional unit, called the *ara* operon, whose expression is directed by a single σ^A -type promoter identified within a 150 bp DNA fragment upstream from the translation start site of *araA*. The *araN*, *araP* and *araQ* gene products are likely components of a binding-protein-dependent transport system and *abfA* most probably encodes an α -L-arabinofuranosidase. In this study we define the promoter region of the *ara* operon and examine its expression and regulation using transcriptional fusions of this operon to the *E. coli lacZ* gene. These results indicate that the *ara* operon is regulated at the transcriptional level because expression from the *ara* promoter is induced by L-arabinose and repressed by glucose.

METHODS

Bacterial strains and growth conditions. The *B. subtilis* strains used in this study are listed on Table 1. *E. coli* DH5 α (Gibco/BRL) was used as a host for all plasmids and *E. coli* DH5 α F' (BRL) for the propagation and amplification of recombinant M13 bacteriophages. *E. coli* strains were grown on LB (Luria-Bertani medium; Miller, 1972). Ampicillin (Ap, 75 μ g ml⁻¹), chloramphenicol (Cm, 15 μ g ml⁻¹), X-gal (40 μ g ml⁻¹) or IPTG (1 mM) were added as appropriate. *B. subtilis* strains were grown on LB, SP medium (Martin *et al.*, 1987) or minimal C medium (Pascal *et al.*, 1971). Cm (5 μ g ml⁻¹), erythromycin (Em, 1 μ g ml⁻¹), kanamycin (Km, 25 μ g ml⁻¹) or spectinomycin (Sp, 50 μ g ml⁻¹) were added as appropriate. Solid medium was made with LB, SP or minimal C medium containing 1.5% (w/v) Bacto Agar (Difco). To test for growth of *B. subtilis* integrant strains on L-arabinose as sole carbon source, strains were plated on minimal C medium containing 0.1% (w/v) L-arabinose. The Ara⁻ phenotype was determined on minimal C medium plates supplemented with 1% (w/v) casein hydrolysate, 0.1% L-arabinose and 1% (w/v) ribitol. To determine specific growth rates, the *B. subtilis* strains were grown in liquid C medium with 0.4% L-arabinose as sole carbon source. The cultures were incubated with aeration by shaking (130 r.p.m.) and cell growth was monitored by OD₆₀₀. For β -galactosidase assays and RNA preparation the *B. subtilis* strains were grown in liquid C medium supplemented with 1% (w/v) casein hydrolysate, and L-arabinose and glucose were added to the cultures when necessary at a final concentration of 0.4% (w/v).

DNA manipulations and sequencing. DNA manipulations were carried out according to Sambrook *et al.* (1989). Enzymes were purchased from commercial suppliers and used according to the manufacturers' instructions. DNA sequencing was performed by the method of Sanger *et al.* (1977) with the Sequenase Kit (T7 DNA polymerase; USB). Sequencing templates were prepared by a combination of subcloning appropriate fragments from pSNL1 and pSNL9 into the polycloning site of M13mp19 or M13mp18 (Yanisch-Perron *et al.*, 1985) and sequential deletion of the recombinant M13 derivatives, by the method of Dale *et al.* (1985), using the Cyclone Biosystem Kit (International Biotechnologies Inc.). The DNA sequence was determined on both strands and across all the restriction sites used for subcloning. The primer 5' CCTCTTCGCTATTACGCC 3', complementary to the coding sequence of *lacZ*, was used to sequence the transcriptional *lacZ* fusions.

Plasmid constructions. pSNL7 was constructed by subcloning a 959 bp *SmaI*-*PstI* DNA fragment (nt 938-1897, Fig. 1) from pSNL1 (Sá-Nogueira & Lencastre, 1989) between the *SmaI* and *PstI* sites of the integrational vector pJM783 (Perego, 1983). To construct pSS2, we digested pSNL1 (Sá-Nogueira & Lencastre, 1989) with *HindIII* and *XhoI* and cloned a purified fragment of 965 bp (nt 3815-4780, Fig. 1) between the *HindIII* and *SalI* sites of the integrating vector pJH101 (Ferrari *et al.*, 1983). pTN10 was obtained by subcloning a 789 bp *HindIII*-*HincII* DNA fragment (nt 6545-7334, Fig. 1) from pSS3 between the *HindIII* and *EcoRV* sites of the integrational vector pJH101 (Ferrari *et al.*, 1983). pTN14 was constructed by subcloning the 678 bp *SmaI*-*BglII* DNA fragment (nt 8242-8920, Fig. 1) from pTN13 between the *BamHI* and *SstI* (fill-in) sites of pJM783 (Perego, 1993). pSNL10 was obtained by subcloning a 1.7 kb *EcoRI*-*HincII* fragment (nt 2681-4416, Fig. 1) from pSNL1 (Sá-Nogueira & Lencastre, 1989) between the *EcoRI* and *SmaI* sites of pMK4 (Sullivan *et al.*, 1984).

Table 1. *B. subtilis* strains

Strain*	Genotype	Phenotype	Source†
168T ⁺	Prototroph	Ara ⁺	F. E. Young
BR151	<i>metB10 lys3 trpC2</i>	Ara ⁺	F. E. Young
IQB100	<i>araB'</i> ::pSNL7(<i>araB</i> -cat <i>lacZ</i>)	Cm ^r Ara ⁻	pSNL7 → 168T ⁺
IQB101	<i>araB'</i> :: <i>lacZ erm</i>	LacZ ⁺ Em ^r Ara ⁻	pSNL11‡ → 168T ⁺
IQB102	<i>araB'</i> :: <i>erm lacZ</i>	LacZ ⁻ Em ^r Ara ⁻	pSNL12‡ → 168T ⁺
IQB103	<i>araA'</i> ::pSNL13 (<i>araA</i> - <i>lacZ cat</i>)	LacZ ⁺ Cm ^r Ara ⁻	pSNL13 → 168T ⁺
IQB104	<i>araA'</i> ::pSNL14 (<i>araA</i> -cat <i>lacZ</i>)	LacZ ⁻ Cm ^r Ara ⁺	pSNL14 → 168T ⁺
IQB202	<i>araL'</i> ::pSS2 (<i>araL</i> -amp cat)	Cm ^r Ara ⁺	pSS2 → 168T ⁺
IQB204	<i>araN'</i> ::pTN10 (<i>araN</i> -cat amp)	Cm ^r Ara ⁺	pTN10 → 168T ⁺
IQB205	<i>araQ'</i> ::pTN14 (<i>araQ</i> - <i>lacZ' cat</i>)	LacZ ⁻ Cm ^r Ara ⁺	pTN14 → 168T ⁺
IQB206	Δ <i>araL</i> - <i>abfA</i> :: <i>spc</i>	Sp ^r Ara ⁺	pSN22‡ → 168T ⁺

* All strains are derivatives of *B. subtilis* 168T⁺.

† The arrows indicate transformation and point from donor DNA to recipient strain. F. E. Young, University of Rochester New York, USA.

‡ Transformation was carried out with linearized plasmid DNA.

pSNL11 and pSNL12 were obtained as follows. A 4.5 kb *Bam*HI-*Hind*III (fill-in) fragment extracted from pMC11 (Debarbouille *et al.*, 1990), containing *lacZ* and *erm* from pTV32 (Perkins & Youngman, 1986), was subcloned in both orientations at the unique *Eco*RV restriction site (nt 3214, Fig. 1) of pSNL10. pSNL13 and pSNL14 were obtained by subcloning a 470 bp *Dra*I-*Eco*RV DNA fragment (nt 82-552, Fig. 1) from pSNL9 at the unique *Sma*I site of the integrational vector pJM783 (Perego, 1993) in both orientations. pSNL13 contains *lacZ* in the same orientation as the *araA* region sequences and pSNL14 contains *lacZ* in the opposite orientation. pSN20 was constructed by cloning the 1.2 kb *Eco*RV-*Hinc*II fragment (nt 3214-4416, Fig. 1) from pSS3 into the *Sma*I site of pAH248 [a pGem-7Zf(+) (Promega) derivative that contains a Km^r gene cloned between its *Xho*I and *Eco*RI sites (A. O. Henriques & C. P. Moran Jr, Emory University School of Medicine, Atlanta, GA, USA, personal communication)]. To obtain pSN21 a 1.7 kb *Eco*RV fragment from pSN5 (nt 10632-about 12332, Fig. 1) was inserted into the *Hinc*II site of pAH250 [a pBluescript SK+ (Stratagene) derivative that contains a Sp^r gene (*spc*) cloned into the *Eco*RV site (A. O. Henriques, B. W. Beall & C. P. Moran Jr, personal communication)]. To construct pSN22, we digested pSN20 with *Pst*I and *Nsi*I and cloned a purified fragment of about 2790 bp, which contains the Km^r gene, in the *Sma*I site of pSN21. pSNL9, pSS3, pTN13 and pSN5 were obtained by cutting chromosomal DNA from *B. subtilis* strains IQB100, IQB202, IQB204 and IQB205 (Table 1) with *Hind*III, *Eco*RI, *Nco*I and *Sma*I, respectively, followed by circularization of the DNA fragments at low concentration.

Bacterial transformation. *B. subtilis* DNA transformations were performed according to the method of Anagnostopoulos & Spizizen (1961). *E. coli* transformations were carried out according to standard methods (Sambrook *et al.*, 1989).

β -Galactosidase assays. Strains of *B. subtilis* harbouring transcriptional *lacZ* fusions were grown in 75 ml C medium supplemented with 1% casein hydrolysate. During early exponential phase (OD₆₀₀ = 0.11-0.15) 25 ml of the culture was transferred to two different flasks and L-arabinose at a final concentration of 0.4% or both L-arabinose and glucose each at a final concentration of 0.4% were added. At this time,

t₀, 100 μ l aliquots of cell culture were collected, harvested and stored at -70 °C overnight. Exponential growth of the three cultures was followed by measuring OD₆₀₀ and at 30 min intervals, 100 μ l of cell culture samples was removed and stored at -70 °C until the cultures reached an OD₆₀₀ = 0.7-0.8, which corresponds to growth for at least 2.5 generations in the presence of the inducer. The cells were resuspended in 1 ml Z buffer (Miller, 1972) and two drops of chloroform plus one drop of 0.1% SDS were added and mixed vigorously for 10 s on a table top vortex apparatus. β -Galactosidase activity was determined as described by Miller (1972) using the substrate ONPG.

RNA preparation, Northern blotting and primer extension analysis. *B. subtilis* 151 or 168T⁺ cells were grown in C medium supplemented with 1% casein hydrolysate in the presence and absence of L-arabinose at a final concentration of 0.4%. Cells were harvested during late exponential phase (OD₆₀₀ ~ 0.9) and RNA prepared as described by Igo & Losick (1986). For Northern blot analysis, 2.5-10 μ g total RNA was run in 1.0-1.2% (w/v) agarose/formaldehyde and transferred to positively charged nylon membranes (Hybond-N+, Amersham) according to standard methods (Sambrook *et al.*, 1989). Size determination was done using an RNA ladder (0.24-9.5 kb; Gibco/BRL). The probes were labelled using the Multiprime random-prime DNA labelling system from Amersham and [α -³²P]dATP [3000 Ci mmol⁻¹ (111 TBq mmol⁻¹)]. Primer extension analysis was performed as described by Sambrook *et al.* (1989). The two synthetic oligonucleotides used in primer extension experiments were primer A (5' GAAGCATGTAACTGCCCC 3'), complementary to nt 216-234 (Fig. 1), and primer B (5' CCAGCGTCTCTCCCCC 3'), complementary to nt 283-300 (Fig. 1). The two oligonucleotides were used in separate experiments to rule out the possibility of primer-specific artifacts. A total of 10 ng of primer was used in the labelling reaction mixed with 25 μ g RNA, denatured by heating to 85 °C for 10 min and annealed by incubation at 42 °C for 3 h. The oligonucleotide primer was extended using 15 units of avian myeloblastosis virus reverse transcriptase for 2 h at 37 °C, as described by

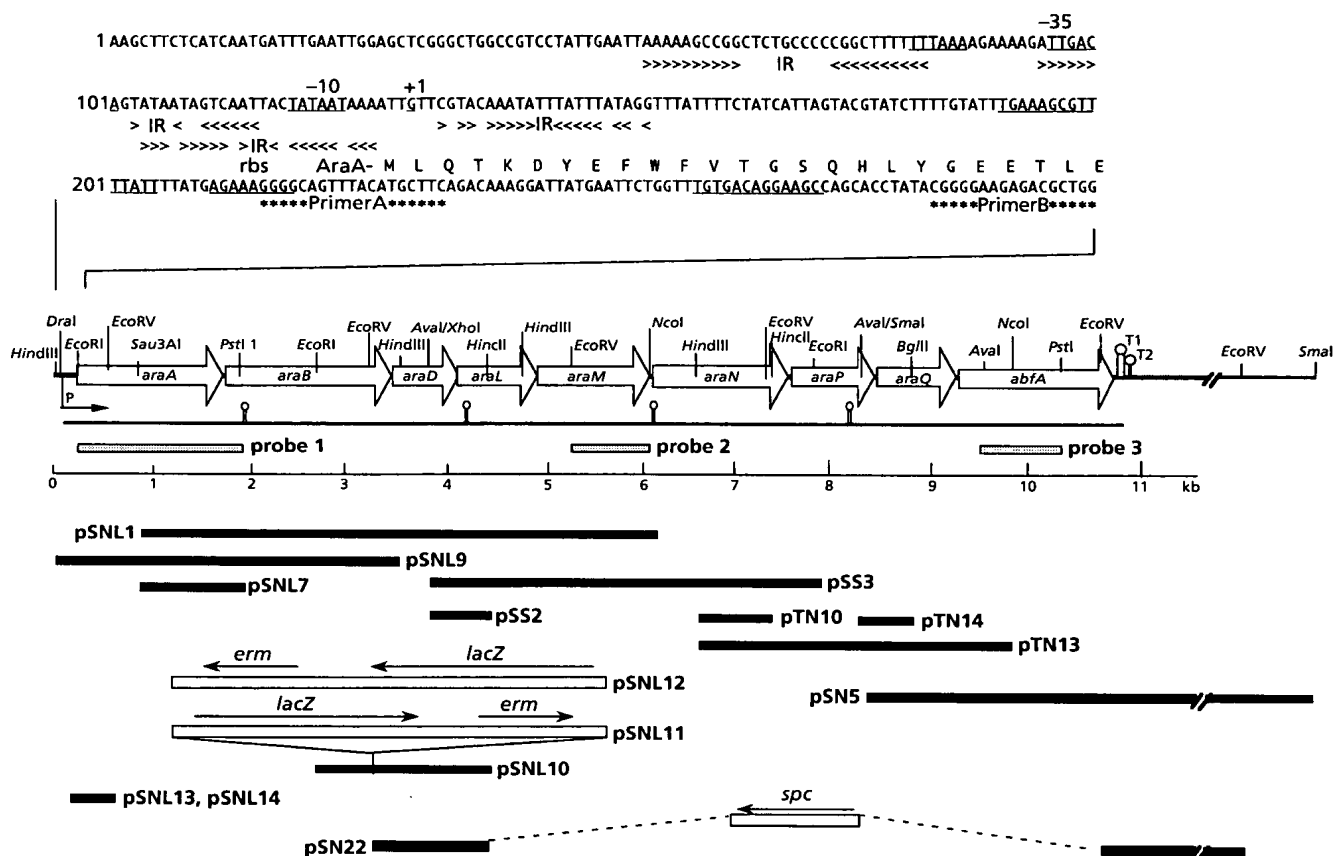


Fig. 1. Physical and genetic map of the *ara* region of the chromosome. The location and direction of transcription of the nine ORFs (*araA*, *B*, *D*, *L*, *M*, *N*, *P*, *Q* and *abfA*), predicted from the analysis of the nucleotide sequence, are indicated by arrows. The promoter (P) of the *ara* operon, defined by primer extension, is located upstream from *araA* and the two regions of dyad symmetry (T1 and T2) that could represent the terminators of the *ara* transcriptional unit are located downstream from *abfA*. Relevant restriction sites are given in the partial restriction map. The region to the right of the *EcoRI* site (position 11755) is not drawn to scale. Immediately below the physical map the *ara* operon transcript is schematically shown and putative secondary structures of the mRNA are indicated by stem-loop structures. The grey boxes, below the physical map, represent the three fragments used as probes for Northern analysis of the *ara* transcripts and the black boxes represent the extent of the inserts in the indicated plasmids. The sites of different insertion-deletion mutations resulting from replacement of wild-type sequences, by double cross-over events (confirmed by Southern blot analysis, data not shown), with *in vitro*-engineered fragments of the *ara* region, present in plasmids pSNL11, pSNL12 and pSN22, are also shown. Plasmids pSNL7, pSS2, pTN10, pTN14, pSNL13 and pSNL14 were integrated into the host chromosome by means of a single cross-over (Campbell-type) recombinational event that occurred in the region of homology (confirmed by Southern blot analysis, data not shown). The *ara* operon promoter nucleotide sequence of the non-transcribed strand is shown in the 5'–3' direction above the physical map. The predicted N-terminal region of the polypeptide encoded by *araA* is given in single letter code. The transcription start site (+1), defined by primer extension analysis, the –35 and –10 regions of the promoter and the putative ribosome binding site (rbs) are underlined. Convergent arrows represent different regions of dyad symmetry (IR) and the complementary sequence of the two primers A and B, used in primer extension analysis are represented below the sequence. The two putative catabolic-repression-associated sequences (positions 191–204 and 260–273) are underlined.

Sambrook *et al.* (1989). Analysis of the extended products was carried out on 7.5% polyacrylamide urea gels.

Computer analysis. Amino acid sequences were deduced from the nucleotide sequence using DNASIS V2.0 (Hitachi Software Engineering, 1991). The GenBank and EMBL databases were accessed using the GCG package of sequence analysis software (Genetics Computer Group, Madison, Wisconsin, USA).

RESULTS

Insertional inactivation of *araB* and cloning of an intact copy of *araA*

The location of the *araA* locus at one end of the cloned fragment in pSNL1 (Fig. 1), together with the absence of *araA* complementation with pSNL1, suggested that only

Table 2. Percentage amino acid identity between the predicted sequences of the Ara proteins and similar proteins

<i>B. subtilis</i> AraA protein	Homologue (species/accession no.)*	Function	Identity (%)	Amino acid overlap
AraA	AraA (<i>E. coli</i> /M15263)	L-Arabinose isomerase	52.9	495
	AraA (<i>Sal. typhimurium</i> /M11047)	L-Arabinose isomerase	52.9	495
AraB	AraB (<i>E. coli</i> /M15263)	L-Ribulokinase	25.7	552
	AraB (<i>Sal. typhimurium</i> /M11045)	L-Ribulokinase	30.6	350
AraD	AraD (<i>E. coli</i> /M15263)	L-Ribulose-5-P 4-epimerase	57.1	231
	AraD (<i>Sal. typhimurium</i> /M11046)	L-Ribulose-5-P 4-epimerase	58.0	205
AraL	NagD (<i>E. coli</i> /X14135)	Unknown	25.5	251
AraN	LacE (<i>Agrobacterium radiobacter</i> /X66596)	Lactose-binding protein	26.2	302
	MalX (<i>Streptococcus pneumoniae</i> /L08611)	Maltose-binding protein	24.1	345
	AmyE (<i>Thermoanaerobacterium thermosulfurigen</i> /M57692)	Starch-binding protein	21.7	369
AraP	LacF (<i>Agrobacterium radiobacter</i> /X66596)	Membrane protein	29.6	284
	UgpA (<i>E. coli</i> /X13141)	Membrane protein	26.2	286
	AmyD (<i>Thermoanaerobacterium thermosulfurigen</i> /M57692)	Membrane protein	25.4	284
AraQ	MalC (<i>Streptococcus pneumoniae</i> /L08611)	Membrane protein	25.2	298
	LacG (<i>Agrobacterium radiobacter</i> /X66596)	Membrane protein	32.7	254
	UgpE (<i>E. coli</i> /X13141)	Membrane protein	22.9	279
	AmyC (<i>Thermoanaerobacterium thermosulfurigen</i> /M57692)	Membrane protein	28.2	262
AbfA	MalD (<i>Streptococcus pneumoniae</i> /L08611)	Membrane protein	25.6	262
	AbfA (<i>Streptomyces lividans</i> /U04630)	α -L-Arabinofuranosidase	52.6	500

part of *araA* was present in this plasmid (Sá-Nogueira & Lencastre, 1989). To clone the entire *araA* gene, plasmid pSNL7 (Fig. 1) was integrated, as single copy, into the *B. subtilis* 168T⁺ chromosome at the *araA* and *araB* region of homology. This procedure causes disruption of the transcriptional unit and the structure of the resulting strain IQB100 that was unable to grow on minimal medium containing L-arabinose as sole carbon source, confirming the polar effect of the insertion on the genes located downstream from *araA*. Furthermore, strain IQB100 showed resistance to ribitol in the presence of L-arabinose on minimal medium plates supplemented with 1% casein hydrolysate. In *B. subtilis* (Paveia & Archer, 1992a), like in *E. coli* (Katz, 1970), these results indicate a defective *araB*. Chromosomal DNA from IQB100 was used to rescue the entire *araA* gene and its upstream region (see Methods). The structure of the recircularized plasmid, pSNL9, was analysed and it contains a 950 bp fragment of DNA upstream from the previously cloned DNA in plasmid pSNL7 (Fig. 1).

Cloning of the chromosomal region extending downstream from *araD*

To clone the region located downstream from *araD*, an integrational plasmid, pSS2, carrying sequences of *araD* and *araL* (Fig. 1), was transformed into the wild-type strain 168T⁺. After integration as single copy, the resulting strain IQB202 presented an Ara⁺ phenotype although the growth on minimal medium plates with L-arabinose as sole carbon source was slower than that observed with the wild-type strain 168T⁺ (see Discussion

below). The digestion of total chromosomal DNA from IQB202 followed by circularization of the fragments yielded plasmid pSS3 that includes a 3.0 kb insert located downstream to the fragment cloned in pSS2 (Fig. 1). To obtain a fragment that would contain the downstream region from *araN*, we performed a second chromosome walking step, using integrational plasmid pTN10 (Fig. 1). This procedure created plasmid pTN13 that carried an additional 3.2 kb of DNA adjacent to the previously cloned fragment in plasmid pTN10 (Fig. 1). Strain IQB204, which resulted from the integration of plasmid pTN10 (Fig. 1) into the chromosome of the wild-type strain 168T⁺ showed a Ara⁺ phenotype similar to that seen with IQB202. A third chromosome walking step rightwards from pTN13, using integrational plasmid pTN14 (Fig. 1), isolated a 4.7 kb *Sma*I fragment (plasmid pSN5). Plasmid pTN14, when integrated into the chromosome of strain IQB205 as single copy, caused an Ara⁺ phenotype. The structure of the inserts in pSS3, pTN13 and pSN5 was compared to that of the corresponding areas of chromosomal DNA by Southern blot analysis (data not shown) and the results revealed that no detectable rearrangement occurred during the cloning process.

DNA sequence and deduced products of *ara* genes

Appropriate restriction fragments, selected on the basis of the physical maps of pSNL1, pSNL9, pSS3, pTN13 and pSN5, were subcloned into M13mp18 and M13mp19 and used as templates to determine the nucleotide sequence of the 11 kb DNA region shown in

Fig. 1. Sequence analysis revealed the presence of nine ORFs; the first three, by their position in the sequenced fragments of pSNL1 and pSNL9 and according to our previous results (Sá-Nogueira & Lencastre, 1989), were identified as *araA*, *araB* and *araD* (Fig. 1). *araA*, *araB* and *araD* could encode 496, 560 and 229 aa products of 56.2, 60.9 and 25.7 kDa, respectively. The six ORFs found downstream from *araD*, here named *araL*, *M*, *N*, *P*, *Q* and *abfA* (Fig. 1), of 269, 394, 433, 313, 281 and 499 codons, are capable of encoding putative products of 29, 43.1, 48.7, 35, 31.8 and 57 kDa, respectively. All ORFs are preceded by strong ribosome binding sites with the exception of *araL* which possesses a weak ribosome binding site. The intercistronic regions are very short and overlaps were observed between the *araD* and *araL* coding sequences, and between *araL* and *araM*, suggesting translational coupling. Two potential hairpin-loop structures, situated next to the UAA stop codon of *abfA* (T_1 and T_2 , Fig. 1, with ΔG values of -27.4 and -18.7 kcal mol $^{-1}$, respectively, according to Tinoco *et al.*, 1973), probably correspond to transcription terminators. The absence of transcriptional signals among the nine coding regions suggested that they form a large operon transcribed from a promoter (described below) positioned 104 nt upstream from the *araA* start codon (Fig. 1).

Comparison of the primary structures of the products predicted to be encoded by the *ara* genes with GenBank sequences revealed significant similarities with other bacterial proteins of known function and the results are summarized in Table 2. The putative product of *araM*, a hydrophilic protein, did not show any significant similarity. The *araA*, *araB* and *araD* gene products exhibited a high level of identity to the L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase, respectively, of *E. coli* and *Salmonella typhimurium*. The product of *araL*, a hydrophilic protein, displayed similarity to the *nagD* gene product of unknown function, which belongs to the *nag* regulon of *E. coli* involved in the metabolism of N-acetyl glucosamine (Plumbridge, 1989). The N-terminal region of the predicted sequence also shared 28.1% and 29.2% identity (over 121 and 106 aa, respectively, data not shown) with two 4-nitrophenylphosphatases, Pho2 and Pho13, from *Schizosaccharomyces pombe* (Yang *et al.*, 1991) and *Saccharomyces cerevisiae* (Kaneko *et al.*, 1989), respectively.

The predicted primary structure of *araN* showed similarity to known sugar-binding proteins that belong to the family of binding-protein-dependent transport systems (Table 2). Although the identity was not very high, there was significant sequence conservation within the N-terminal region of these proteins which display a signature sequence, according to Tam & Saier (1993). On the basis of this signature sequence (Fig. 2a) *AraN* can be included in the cluster 1 binding proteins (according to Tam & Saier, 1993), together with the above-mentioned proteins involved in the transport of malto-oligosaccharides and multiple sugars. The hydrophathy profile of *AraN* indicated that it is mainly a

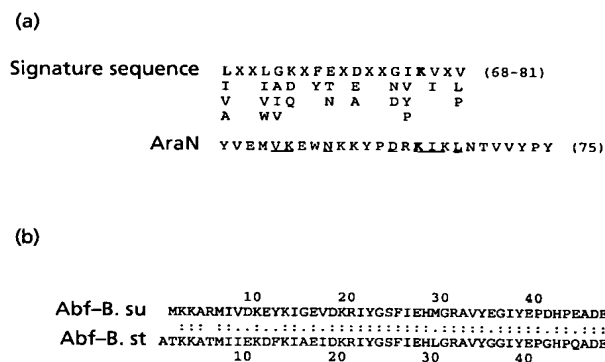


Fig. 2. (a) Alignment of a segment of the predicted sequence of the *AraN* protein with the signature sequence of cluster 1 binding proteins, from binding-protein-dependent transport systems, according to Tam & Saier (1993). Numbers in parentheses indicate the positions of the last amino acid residues. The highly conserved lysine residue (K) is in bold and the amino acid residues that match the signature sequence are underlined. (b) Alignment of the N-terminal sequence (deduced from the nucleotide sequence) of *AbfA* from *B. subtilis* (B. su) with the N-terminal sequence of α -L-arabinofuranosidase from *B. stearothermophilus* (B. st). Double dots represent identical amino acids and single dots represent conservative changes.

hydrophilic protein; however its N-terminal region displayed characteristics of signal peptides of secretory precursor proteins: a positively charged N terminus, a hydrophobic core and a sequence, IAGCSA (starting at aa 19), which corresponds to the consensus sequence for the precursors of lipoproteins (reviewed in Hayashi & Wu, 1990).

The predicted products of *araP* and *araQ* exhibited hydrophathy profiles (according to Kyte & Doolittle, 1982) characteristic of integral membrane proteins: six major regions of high hydrophobicity (hydropathic index > 1.0), each composed of at least 20 aa which could be capable of spanning the membrane (Fig. 3a). *AraP* and *AraQ* shared an identity of 19.6% and showed significant similarity with integral cytoplasmic membrane proteins involved in prokaryotic binding-protein-dependent transport systems (Table 2). In common with most of these integral membrane proteins, *AraP* and *AraQ* have a conserved hydrophilic segment (Fig. 3b) at approximately 100 residues from the C terminus with the consensus EAA---G-----I-LP (Dassa & Hofnung, 1985). Furthermore, on the basis of this signature sequence, they can be included in the disaccharide sub-cluster proposed by Saurin *et al.* (1994) together with the above-mentioned proteins involved in the transport of malto-oligosaccharides, multiple sugars and α -glycerol phosphate.

The deduced product of *abfA*, a hydrophilic protein, displays a N-terminal region (Fig. 2b) which resembles a signal peptide of exoproteins (reviewed on Gierasch, 1989 and Nagarajan, 1993): a positively charged N terminus, a hydrophobic core and a potential cleavage site (AV, position 32-33, Fig. 2b). The primary structure of the putative product of *abfA* is closely related to the

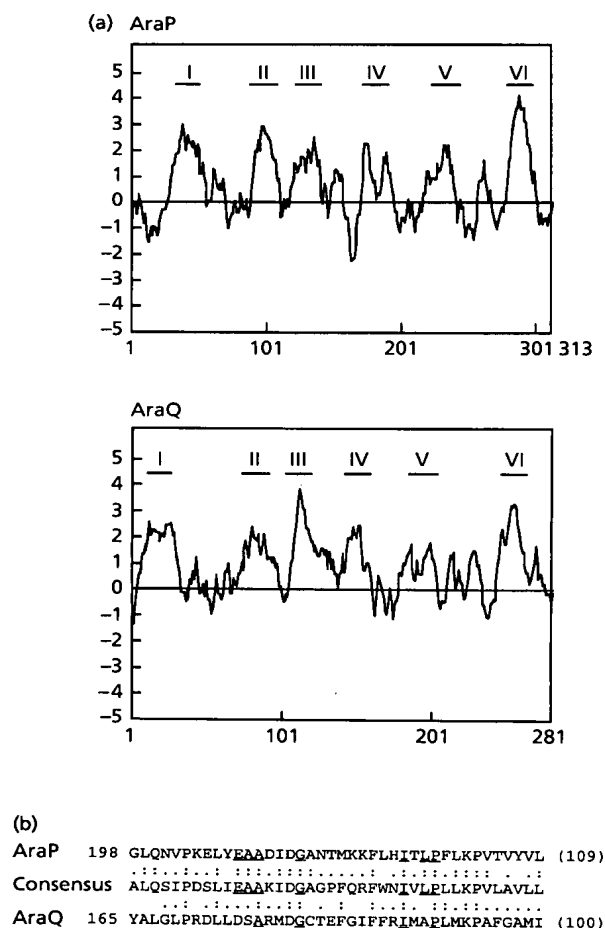


Fig. 3. (a) Hydropathic index for the deduced amino acid sequences of AraP and AraQ according to the algorithm of Kyte & Doolittle (1982). The hydropathy profiles are plotted from the N terminus to the C terminus by averaging hydropathy values over a window of 10 residues. Hydrophobic segments which could correspond to membrane-spanning regions are labelled I–VI. (b) Alignment of a hydrophilic segment, at approximately 100 residues from the C terminus of the predicted sequences of AraP and AraQ, with the consensus sequence for the group of integral cytoplasmic membrane proteins from binding-protein-dependent transport systems (Saurin *et al.*, 1994), which includes permeases involved in the transport of disaccharides and glycerol phosphate. The general consensus for integral membrane proteins from binding-protein-dependent permeases, EAA—G—L—P, where (–) represents any amino acid (Dassa & Hofnung, 1985), is underlined. The distance of the invariant glycine residue from the C terminus is represented in parentheses. Double dots represent identical amino acids and single dots represent conservative changes.

α -L-arabinofuranosidase of *Streptomyces lividans* (Table 2) and the N-terminal region (Fig. 2b) is 74% identical and 96% similar to the sequenced N terminus of purified α -L-arabinofuranosidase from *Bacillus stearo-thermophilus* (Gilead & Shoham, 1995). These observations strongly suggest that *abfA* encodes an α -L-arabinofuranosidase.

RNA transcript analysis of the L-arabinose gene region

Total RNA from cells grown in the presence and absence of L-arabinose was isolated, blotted and hybridized to three different DNA probes (probes 1, 2 and 3, Fig. 1) each specific to one gene of the *ara* region (*araA*, *araM* and *abfA*, respectively). Northern blot analysis (Fig. 4) revealed that *ara* genes are organized in a large polycistronic operon, and that transcripts could be detected only if the cells were grown in the presence of L-arabinose. In addition to a transcript of 11 kb comprising all genes and detected with the three probes, several other signals of different intensities were obtained depending on the probe used (Fig. 4). Using the *araA*-specific probe, we detected five different transcripts of about 8.2, 6.4, 5.8, 4 and 1.9 kb, considering a margin of error of 10–15% for the size determination of transcripts. Two additional transcripts of about 8.2 and 6.4 kb were visualized with the *araM*-specific probe and three hybridization signals were obtained with the *abfA*-specific probe: 8.3, 4.8 and 1.1 kb. Interestingly, stable secondary structures were identified at the corresponding sites within the *araB*, *araL*, *araN* and *araQ* sequences (Fig. 1). The exact nature of these different minor transcripts is unknown but they might be generated by premature transcription termination and/or processing of the multicistronic messenger or RNA degradation. Another possible explanation is the presence of transcription initiation sites located downstream from the promoter defined by primer extension analysis (see below).

The promoter region and transcriptional start site of the *ara* operon

To determine the transcriptional start site of the *ara* operon, total RNA was extracted during the exponential growth of wild-type cultures in the presence and in the absence of L-arabinose. Reverse transcripts were obtained using an end-labelled 17-mer (primer B, Fig. 1), designed to hybridize to part of the *araA* mRNA. A single extension product was detected with RNA isolated from cells grown in the presence of L-arabinose, the size of which suggests that transcription of the *ara* operon starts at a G residue situated 97 nt upstream from the *araA* start codon (Fig. 5). No extension product was seen when RNA was isolated from cells grown in the absence of L-arabinose. The same transcription start point was obtained using a second primer (primer A, Fig. 1) designed to hybridize to part of the mRNA 50 bases upstream from the first primer (Fig. 5). The synthesis of the *ara* operon mRNA is induced by L-arabinose and driven by a strong promoter as evaluated by the intensity of the reverse transcript signal obtained. Situated 7 and 30 bp upstream from the *ara* operon transcription start site are sequences identical to the consensus –35 and –10 regions (TTGACA-17 bp-TATAAT), respectively, of promoters recognized by *B. subtilis* σ^A -containing RNA polymerase (Moran *et al.*, 1982). Sequence analysis of the promoter region revealed the existence of three inverted repeats, putative

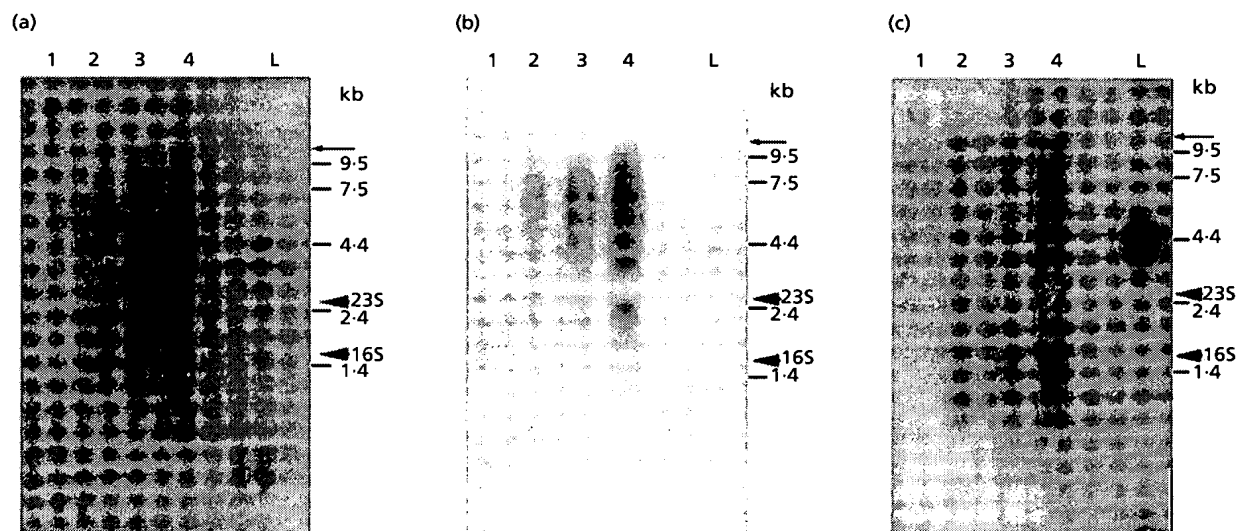


Fig. 4. Northern analysis of the *ara* operon-specific transcripts. Lanes: 1, 10 µg total RNA extracted from the uninduced wild-type strain *B. subtilis* 168T⁺; 2, 3 and 4, 2.5 µg, 5 µg and 10 µg, respectively, of total RNA extracted from the induced wild-type strain *B. subtilis* 168T⁺ grown on L-arabinose (see Methods); L, 4 µg RNA ladder (0.24–9.5 kb; Gibco/BRL). The samples were run in 1% (a, b) and 1.2% (c) agarose formaldehyde denaturing gel. The ³²P-labelled probes used were synthesized from (a) a 1.6 kb *Eco*RI–*Pst*I fragment (position 249–1897, probe 1), (b) a 0.8 kb *Nco*I–*Eco*RV fragment (position 5270–6079, probe 2) and (c) a 0.7 kb *Pst*I–*Ava*I fragment (position 9538–10275, probe 3). The RNA ladder was probed with ³²P-labelled λ DNA and also visualized by staining with ethidium bromide. The transcript of about 11 kb comprising all genes and detected with the three probes is indicated by an arrow.

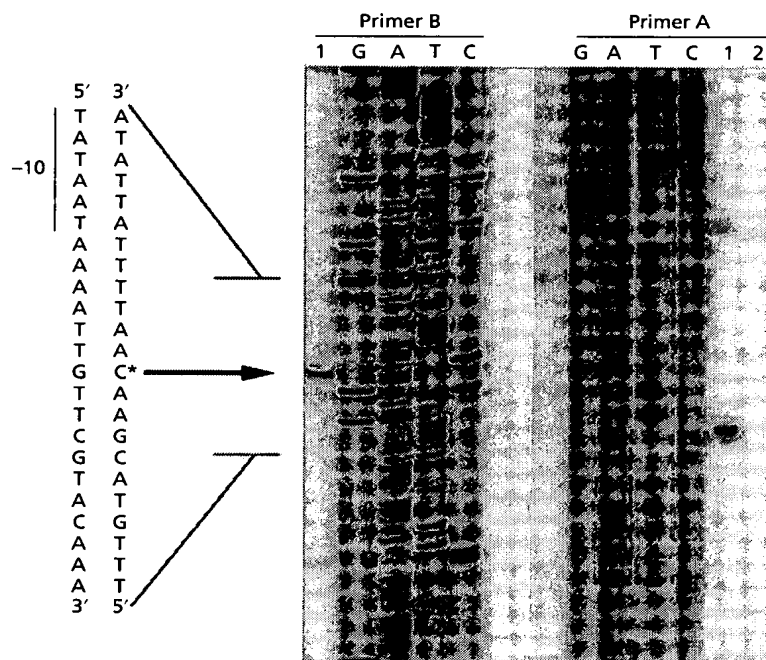


Fig. 5. Primer extension analysis of the *ara* operon promoter. Two radiolabelled oligonucleotide primers, A and B, complementary to two different regions downstream from the *ara*ABD promoter [primer A, 5' GAAGCATGTAACTGCC 3', complementary to a region of *ara*A mRNA located between nucleotides 216 and 234 (Fig. 1) and B, 5' CCAGCGTCTCTCCCG 3', complementary to a region of the *ara*A mRNA located between nucleotides 283 and 300 (Fig. 1)] were hybridized with *B. subtilis* BR151 RNA isolated from exponentially growing cells in the presence (lane 1) or absence (lane 2) of L-arabinose. After extension, the products were analysed by gel electrophoresis, together with a set of dideoxynucleotide chain-termination sequencing reactions using the same primers and a single-stranded M13 DNA template which includes the entire *ara*A gene and an additional 228 bp of its 5' flanking sequence.

operator-like sequences, in the –35 and –10 regions (Fig. 1). A potential hairpin-loop structure with a ΔG value of $-19.2 \text{ kcal mol}^{-1}$ (Tinoco *et al.*, 1973), centred

27 bp upstream from the –35 region (Fig. 1), probably corresponds to a transcription terminator of a gene located upstream from the cloned DNA fragment.

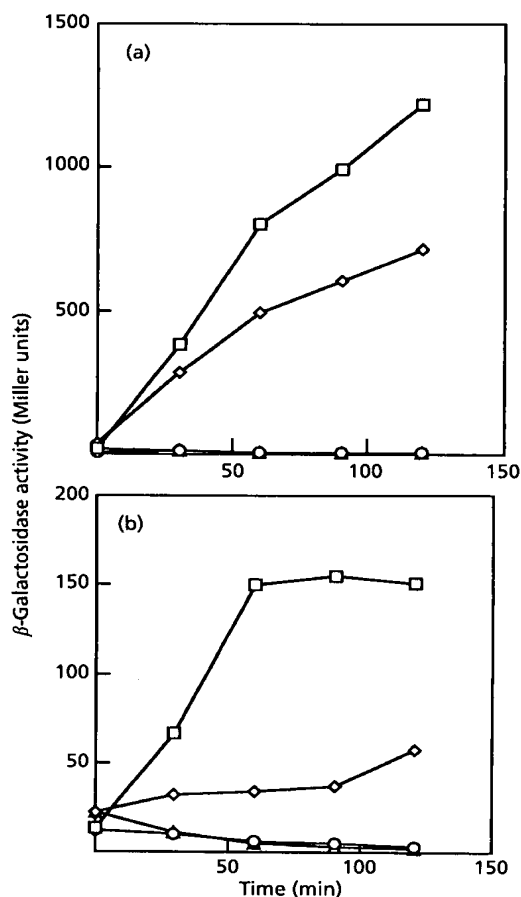


Fig. 6. Expression of the *ara* operon measured by determination of the levels of β -galactosidase activity (Miller units) present in exponentially growing cells. Strains of *B. subtilis* harbouring transcriptional *lacZ* fusions were grown on minimal C medium supplemented with 1% casein hydrolysate and either (a) 0.4% L-arabinose or (b) 0.4% L-arabinose plus 0.4% glucose (see Methods). Time is expressed in minutes after induction. ◇, IQB101 (*araB'*-*lacZ erm*; *Ara*⁻ *Em*^r *LacZ*⁺); □, IQB103 (*araA'*-*lacZ cat*; *Ara*⁺ *Cm*^r *LacZ*⁺); △, IQB102 (*araB'*-*erm lacZ*; *Ara*⁻ *Em*^r *LacZ*⁻; negative control); ○, IQB104 (*araA'*-*cat lacZ*; *Ara*⁺ *Cm*^r *LacZ*⁻; negative control). For each strain the results represent the mean, in Miller units, of two independent experiments.

Expression of the *ara* operon is induced by L-arabinose and repressed by glucose

To study the regulation of expression of the operon we constructed transcriptional *lacZ* fusions at this locus. The replicative plasmids pSNL11 and pSNL12, carrying *lacZ* and *erm* (Fig. 1), were linearized and used separately to transform the wild-type 168T⁺ strain. This resulted in the integration of *lacZ* and *erm* into the chromosome at the *araB* locus. The resulting strains, IQB101 (*araB'*-*lacZ erm*) and IQB102 (*araB'*-*erm lacZ*), were unable to grow on L-arabinose as sole carbon source, which confirmed the insertional inactivation of

araB. The integrational plasmids pSNL13 and pSNL14, carrying the same DNA fragment in opposite orientations (Fig. 1), were integrated as single copy into the chromosome of the wild-type strain 168T⁺. The resulting strains, IQB103 (*araA'*-*lacZ cat*) and IQB104 (*araA'*-*cat lacZ*), respectively, displayed an *Ara*⁺ phenotype because the integration was not disruptive. The *LacZ* phenotype of the four strains was tested on minimal C medium plates supplemented with 1% casein hydrolysate and X-Gal. Upon addition of L-arabinose to the medium, strains IQB101 and IQB103 presented a dark blue phenotype, whereas those of IQB102 and IQB104 remained white, confirming that the expression of the operon is driven from a promoter located upstream from *araA* and induced by L-arabinose. Furthermore, addition of other pentoses such as D-xylose and D-ribose failed to induce a *LacZ*⁺ phenotype in strain IQB103. The regulation of *ara* operon expression was examined in cultures during mid-exponential phase in minimal C medium supplemented with 1% casein hydrolysate as described in Methods. The levels and patterns of *lacZ* expression in IQB101 (*araB'*-*lacZ erm*; *Ara*⁻ *LacZ*⁺), IQB103 (*araA'*-*lacZ cat*; *Ara*⁺ *LacZ*⁺), IQB102 (*araB'*-*erm lacZ*; *Ara*⁻ *LacZ*⁻; negative control) and IQB104 (*araA'*-*cat lacZ*; *Ara*⁺ *LacZ*⁻; negative control) determined in the presence of L-arabinose and L-arabinose plus glucose are shown in Fig. 6. When the four strains were grown in the absence of inducer, the level of accumulated β -galactosidase activity, at time *t* = 120 min, was 4.4, 4.8, 2.8 and 1.8 Miller units, respectively. In the presence of L-arabinose the pattern of expression observed in strains IQB101 (*araB'*-*lacZ*; *Ara*⁻) and IQB103 (*araA'*-*lacZ*; *Ara*⁺) was very similar (Fig. 6) but the levels of accumulated β -galactosidase activity in the *araB* null mutant were less than 60% relative to the wild-type strain (discussed below). Addition of glucose reduced the level of expression to less than 12% in both *Ara*⁺ and *Ara*⁻ backgrounds (Fig. 6). These data demonstrate that L-arabinose is an inducer which stimulates the expression of the *ara* operon at the transcriptional level and transcription is subjected to catabolite repression by glucose. Furthermore, the prediction that the expression of the *ara* operon is driven from a strong promoter, made on the basis of the intensity of the reverse transcript signal observed in primer extension analysis, was confirmed when β -galactosidase activity was measured in strain IQB103 (*araA'*-*cat lacZ*; *Ara*⁺).

araL, *M*, *N*, *P*, *Q* and *abfA* are not required for L-arabinose utilization

Strains IQB202 and IQB204 in which the integration of plasmids pSS2 and pTN10, respectively, interrupted the transcription unit at *araL* and *araN* (Fig. 1), exhibited an *Ara*⁺ phenotype, however, their growth on minimal medium plates with L-arabinose as sole carbon source was slightly slower than the one observed with the wild-type strain 168T⁺. This phenotype was not observed with strain IQB205 in which pTN13 disrupted the operon at the end of *araQ*. To confirm that *araL*, *M*, *N*,

P, *Q* and *abfA* are not required for L-arabinose utilization, we constructed a deletion in the region downstream from *araD* by replacing *in vitro* the wild-type sequences of *araL*, *M*, *N*, *P*, *Q* and *abfA* with a *Sp*^r cassette and then using it to replace the corresponding chromosomal sequences (see Methods). Plasmid pSN22 (Fig. 1) was linearized and used to transform the wild-type strain 168T⁺ *Sp*^r. The resulting strain IQB206, was Km^s which indicated that the *Sp*^r phenotype was the result of a double cross-over event that occurred on both sides of the cassette inserted between the *araL* and *abfA* sequences (Fig. 1). This mutant strain was able to grow on minimal medium plates with L-arabinose but displayed a phenotype even more drastic than the one exhibited with strains IQB202 and IQB204. To quantify this observation we determined the specific growth rate of the deletion-insertion mutant and the wild-type strain in liquid minimal C medium with L-arabinose as sole carbon source, as described in Methods. The doubling time of strain IQB206 was 1.8-fold higher than the wild-type strain 168T⁺, 193.4 ± 7.2 and 107.7 ± 3.6 min (means of three independent experiments \pm SEM), respectively. These results confirmed that the genes located downstream from *araD* in the operon are not essential for L-arabinose utilization, however their absence in the deletion mutant affects the specific growth rate in minimal medium with L-arabinose as the sole carbon source when compared to the wild-type strain.

DISCUSSION

In this study we have described a new catabolic operon involved in the utilization of L-arabinose in *B. subtilis*, which we designated *ara*. The arabinose metabolic genes *araA*, *araB* and *araD*, encoding L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase, respectively, were cloned previously and by complementation experiments the products of *araB* and *araD* were shown to be functionally homologous to their *E. coli* counterparts (Sá-Nogueira & Lencastre, 1989). These genes, whose inactivation leads to an Ara⁻ phenotype, were found to be the first three ORFs of a nine cistron transcriptional unit whose total length is 11 kb. To our knowledge this operon is the largest catabolic operon described in *B. subtilis*. As expected from the occurrence of genetic complementation, the deduced products of *araA*, *araB* and *araD* from *B. subtilis* display a very high level of identity to the corresponding enzymes from *E. coli* and *Sal. typhimurium*, which indicates that this metabolic pathway was fundamentally conserved during evolution. In *B. subtilis* the metabolic gene order, *araABD*, coincides with the order of the enzymic steps carried out by the proteins they encode. This order is different from the one found in the operons of the *Enterobacteriaceae* members *E. coli* and *Sal. typhimurium*, *araBAD*, so it seems that the three genes did not act as a unitary block in the evolution of the eubacterial *ara* genes.

The six ORFs found downstream from *araD*, here named *araL*, *M*, *N*, *P*, *Q* and *abfA*, are not required for L-arabinose utilization. This was shown in a mutant

strain, IQB206, bearing a deletion in the region downstream from *araD* comprising all genes. The function of *araL* and *araM* is unknown. The putative product of *araM* did not show any significant similarity with other bacterial proteins of known function and the weak similarities displayed by *araL* did not suggest any particular function. Interestingly, the N-terminal sequence of *araL* shares an identity of 18.7% over 193 residues with the C-terminal sequences of *araM* (data not shown). The primary sequences of the products of *araN*, *araP* and *araQ* strongly suggest that they have a similar function to that of a superfamily of membrane-bound nutrient transport systems (Higgins *et al.*, 1990). Sequence similarities to known import proteins and the organization of the genes in the operon revealed the presence of three components of these transport systems. Firstly, the N terminus of AraN has a predicted signal peptide and sequences typical of Gram-positive lipoproteins (IAGCSA, starting at aa 19). We therefore suggest that AraN might be anchored in the cytoplasmic membrane via an amino-lipid group (Gilson *et al.*, 1988; Perego *et al.*, 1991). Secondly, *araP* and *araQ* gene products, as other characterized integral cytoplasmic membrane proteins, have hydropathy profiles which are virtually superimposable and some of their residues are apparently conserved (Fig. 3). Finally, *araN*, *araP* and *araQ* belong to the same operon and the ligand-specific binding protein, AraN, is encoded by the promoter-proximal gene, a situation common to these systems. In *B. subtilis* the phosphotransferase system is not involved in the transport of L-arabinose into the cell (Gay *et al.*, 1973). Therefore, it is tempting to propose that AraN, AraP and AraQ are components of a high affinity transport system for L-arabinose. However, no evident ATP-binding protein connected with energy coupling of the transport system was found in the operon.

The transport of L-arabinose across the *E. coli* cytoplasmic membrane requires the expression of either the high-affinity transport operon, *araFGH*, a binding-protein-dependent system (Horazdovsky & Hogg, 1989; Kolodrubetz & Schleif, 1981) or the low-affinity transport operon, *araE*, a proton symporter (Novotny & Englesberg, 1966). The existence of two parallel uptake systems thwarts usual genetic attempts to isolate mutants defective in either of the transport systems. The Ara⁺ phenotype displayed by the *B. subtilis* deletion-insertion mutant strain IQB206 (Δ *ara-abfA::spc*) together with the 1.8-fold increase in doubling time observed on liquid minimal medium with L-arabinose as the sole carbon source, relative to the wild-type strain, is typical of a transport mutant when the micro-organism has alternative transport systems for the same substrate. An additional explanation for this phenotype observed in the deletion-insertion mutant is that insertion of *spc* might result in a less stable mRNA encoding *araABD*, leading to decreased amounts of their products. Interestingly, the primary structure of AraP and AraQ showed weak similarity with AraH, the integral cytoplasmic membrane protein from *E. coli*, and the same result was observed between AraN and AraF, the *E. coli*

arabinose binding protein (data not shown). Furthermore, on the basis of their signature sequences, AraN, AraP and AraQ can be included in the disaccharide sub-cluster (Figs 2 and 3) together with proteins involved in the high-affinity transport of malto-oligosaccharides and multiple sugars. *B. subtilis* secretes three enzymes involved in the degradation of L-arabinose polymers, an endo-arabanase and two arabinosidases, and the purified endo-arabanase has been shown to be capable of releasing arabinosyl oligomers from plant cell walls (Kaji & Saheki, 1975; Weinstein & Albersheim, 1979). To account for these observations a wider substrate range, L-arabinose and/or L-arabinose oligomers, for the *B. subtilis* AraN binding protein is suggested. The last gene of the *ara* operon, *abfA*, probably encodes a α -L-arabinofuranosidase, based on the strong similarity observed between the primary structure of its putative product and other bacterial arabinosidases. Whether this enzyme is extracellular or intracellular is unknown.

Expression of the *ara* operon is induced by L-arabinose and driven by a promoter located upstream of *araA*. This has been demonstrated in this study by Northern blotting and primer extension analysis. Examination of the *ara* operon promoter reveals -35 and -10 sequences, relative to its transcriptional start site (shown in Fig. 1), separated by an optimal spacing of 17 bp, identical to the consensus sequences derived from the analysis of many σ^A -dependent promoters (Moran *et al.*, 1982). These sequences were shown to be important for the interaction of σ^A with their cognate promoters (reviewed in Moran, 1993). The presence of a strong promoter raises the possibility that transcription of *ara* is negatively regulated like in other well characterized *B. subtilis* catabolic operons, such as *xyl* (Gärtner *et al.*, 1992) and *gnt* (Fujita & Fujita, 1987); in fact the product of *araC* recently cloned, is a negative regulator of the *ara* operon (I. Sá-Nogueira & L. J. Mota, unpublished). To characterize the regulation of *ara* expression in greater detail we constructed transcriptional fusions of the *ara* promoter to the *E. coli lacZ* gene in Ara⁺ and Ara⁻ strains. The induction by L-arabinose in the Ara⁺ background was approximately 100-fold and the pattern of expression observed in Ara⁻ and Ara⁺ strains was very similar. Interestingly however, the levels of accumulated β -galactosidase activity in the Ara⁻ background were less than 60% of the fully induced level in the wild-type strain. Since in this strain the *ara* transcription unit is interrupted at the level of *araB* (Fig. 1), and a role in the transport of L-arabinose was proposed for the downstream genes *araN*, *araP* and *araQ*, this effect could be due to less accumulated intracellular L-arabinose which prevents full expression of the *ara* promoter. Another possible explanation is that the products of *araL* and *araM* could stimulate transcription from the *ara* promoter. Addition of glucose reduced the level of expression to less than 12% in both Ara⁺ and Ara⁻ backgrounds, indicating that repression of the *ara* operon by glucose acts at the transcriptional level.

The regulatory system mediating catabolite repression in *B. subtilis* seems to be accomplished by a negative

regulatory mechanism (reviewed in Hueck & Hillen, 1995; Saier *et al.*, 1996). This evidence is based on the location and the sequences of *cis*-acting sites (CREs) responsible for catabolite repression of several *B. subtilis* genes and operons. Moreover, catabolite repression of most genes regulated via these *cis*-acting sites is also affected by the *trans*-acting factors CcpA, a DNA-binding protein, and HPr, an intermediate in the phosphotransferase sugar transport system. It has been proposed that HPr-Ser-P might interact with CcpA and that this interaction might allow CcpA to bind to the CRE (Deutscher *et al.*, 1994). Strong evidence for this proposal, but also contradictory results, have been obtained recently (Saier *et al.*, 1996; and references therein). CREs of catabolic genes and operons are located either in the promoter regions, where the binding of a regulatory protein probably interferes with transcription initiation, or in the downstream regions (reviewed in Hueck & Hillen, 1995). In the case of the *hut* operon two active CREs were found, one at the promoter and the other within *hutP*, and a looping mechanism involving co-operatively bound CREs has been proposed to interfere with transcription initiation (Wray *et al.*, 1994). Furthermore, the transition-state regulator AbrB is capable of specifically binding to *hut* CRE *in vitro* and an *abrB* null mutation leads to more efficient catabolite repression of some genes in *B. subtilis*, including L-arabinose isomerase. Thus, AbrB has been suggested to compete for binding to CRE with CcpA (Fisher *et al.*, 1994). The promoter region of the *ara* operon contains a sequence very similar to the CRE consensus sequence (TGWNANCGNTNWCA; W = A, T; Weickert & Chambliss, 1990) located between the transcription start site and the translation start site of *araA* (position 191–204, Fig. 1). A second sequence, which shows weak similarity with the CRE consensus sequence was found within *araA* (position 260–273, Fig. 1). Since inducer exclusion does not play a major role in carbon regulation of expression of the *ara* metabolic genes (Sá-Nogueira *et al.*, 1988), as observed in the *hut* operon (Chasin & Magasanik, 1968), it will thus be interesting to investigate the role of CcpA, HPr and AbrB in the catabolite repression of the *ara* operon and whether these sequences are *cis*-acting sites responsible for catabolite repression of the *ara* genes.

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L-Ribulose 5-Phosphate 4-Epimerase from *Aerobacter aerogenes*

EVIDENCE FOR A ROLE OF DIVALENT METAL IONS IN THE EPIMERIZATION REACTION*

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SUMMARY

L-Ribulose 5-phosphate 4-epimerase from *Aerobacter aerogenes* was inactivated by treatment with EDTA and was reactivated to varying extents by the addition of divalent metal ions in the order: $Mn^{++} > Co^{++} > Ni^{++} > Ca^{++} > Zn^{++} > Mg^{++}$. When optimal Mn^{++} was present, the homogeneous enzyme had a specific activity of $70 \mu\text{moles}^{-1} \text{ min}^{-1} \text{ mg}$ of protein at 28° and pH 7.2. This value is about five times greater than that displayed by the crystalline enzyme as isolated and assayed in the absence of added metal ion.

In other mechanistic studies, L-ribulose 5-phosphate 4-epimerase was found to be stable to treatment with sodium sulfite and arsenite in the presence of a thiol compound. It was also stable to sodium borohydride in the presence or absence of substrate. Further, a reaction of tetranitromethane with the enzyme-substrate complex could not be detected. Possible mechanisms for L-ribulose 5-phosphate 4-epimerase are discussed.

L-Ribulose 5-phosphate 4-epimerase from *Aerobacter aerogenes*, which catalyzes the interconversion of L-ribulose-5-P and D-xylulose-5-P, is unique among 4-epimerases in that it neither contains nor requires NAD^+ for catalysis (1). In addition, no evidence was found for the presence of chromophoric substances in the crystalline enzyme, nor did additional cofactors have an influence on the activity (1). In contrast, there is substantial evidence that epimerization by UDP-glucose 4-epimerase involves an oxidation-reduction mechanism utilizing NAD^+ as the electron acceptor and donor (2-6). Thus, if L-ribulose-5-P 4-epimerase catalyzes a similar oxidation-reduction reaction, another as yet unrecognized electron acceptor must perform the function of NAD^+ .

It has also been observed that there is no kinetic isotope effect when D-[4- T]xylulose 5-phosphate is used as substrate (7). This is in contrast to UDP-glucose 4-epimerase where a normal isotope effect is observed (8). In this connection, it may be

significant that the substrates, L-ribulose-5-P and D-xylulose-5-P, are open chain carbohydrates lacking a nucleotide moiety and possessing a carbonyl group two carbon atoms removed from the epimerization site. Undoubtedly, this confers chemical properties on the substrate which are considerably different from those of nucleotide sugars. For these reasons, mechanisms of 4-epimerization of L-ribulose-5-P other than oxidation-reduction have been considered.

The results presented in this paper indicate that L-ribulose-5-P 4-epimerase requires divalent metal ions for activity, and that different divalent metal ions activate to varying extents. In addition, an exploration of a number of mechanistic possibilities involving oxidation-reduction, or carbanion and carbonium ion formation, gave negative results.

MATERIALS

Chemicals—L-Ribulose-5-P was prepared according to the procedure of Anderson (9). Spectro-pure sulfate salts of Mn^{++} , Mg^{++} , Ni^{++} , Zn^{++} , and Co^{++} were obtained from Johnson, Mathley and Co., Ltd. Chloride salts of the divalent metal ions were obtained from Mallinckrodt, Inc. Tris base was obtained from Sigma Chemical Co.

Enzymes—L-Ribulose-5-P 4-epimerase from *A. aerogenes* (constitutive for L-arabinose operon, uracil auxotroph designated "u-i-") and D-xylulose-5-P phosphoketolase from *Leuconostoc mesenteroides* were purified by procedures previously reported (1). A triose phosphate isomerase- α -glycerol phosphate dehydrogenase mixture was obtained from Calbiochem.

METHODS

L-Ribulose-5-P 4-Epimerase Assay—The 4-epimerase was assayed by two methods designated as "continuous" and "two-step." The continuous assay involved the conversion of L-ribulose-5-P to α -glycerol phosphate with the concomitant oxidation of NADH utilizing phosphoketolase, triose phosphate isomerase, and α -glycerol phosphate dehydrogenase as coupling enzymes (1). The two-step assay involved the epimerization of L-ribulose-5-P to D-xylulose-5-P in Tris-Hepes¹ buffer, pH 8.0, in the absence of coupling enzymes. The 4-epimerase was then inactivated by the addition of acetic acid and heating in a boiling water bath for 1 min. The pH was readjusted to 7.0 by the addition of ammonium hydroxide, and an aliquot of the

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¹ The abbreviation used is: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

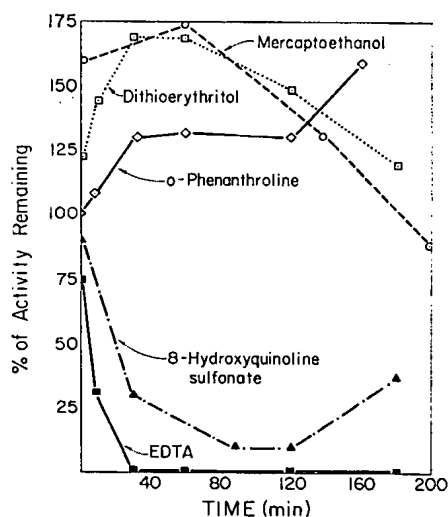


FIG. 1. The effect of metal chelators on enzyme activity. The 4-epimerase preparation, approximately 50% pure, was dialyzed against 0.05 M glycylglycine buffer, pH 8.0, and incubated at room temperature in 0.05 M glycylglycine, pH 8.0, with 1 M mercaptoethanol, 10^{-1} M dithioerythritol, 1 mM *o*-phenanthroline, 8×10^{-2} M 8-hydroxyquinoline sulfonate, or 10^{-3} M EDTA. An aliquot was withdrawn at the times indicated and assayed for 4-epimerase activity in the two-step assay at 37° and pH 8.0, as described under "Methods." Contaminating metal ions were not removed from reagents or glassware used in these assays. All values are expressed as percentage of the activity remaining compared to the activity prior to the addition of the chelator.

assay mixture was assayed for D-xylulose-5-P by measuring the amount of NADH oxidized upon enzymatic conversion of D-xylulose-5-P to α -glycerol phosphate in the presence of phosphoketolase, triose phosphate isomerase, and α -glycerol phosphate dehydrogenase (10). Only small amounts of metal or chelator from the incubation with epimerase was carried over in the analytical step for D-xylulose 5-phosphate. In separate controls it was shown that both the metal ions and chelators used affected the D-xylulose 5-phosphate values less than 10%.

Where indicated, all traces of metal ions were removed from the reagents and glassware used in the first step of the two-step assay. The glassware was soaked overnight in 4 N HCl and then was extensively rinsed with double quartz-distilled water. The buffers used in the assay were passed over Chelex resin and the pH was adjusted with solid Tris base. The L-ribulose-5-P was also passed through a Chelex column, and the pH was adjusted to 6.0 with Tris base prior to use.

One unit of enzyme activity is defined as the amount required to epimerize 1 μ mole of L-ribulose-5-P per min at 28° and pH 7.2.

Protein concentration was determined using $A_{280}:A_{260}$ ratio method of Warburg and Christian (11).

Removal of EDTA by Chromatography on Sephadex G-25—EDTA was removed by passing the enzyme through a Sephadex G-25 (0.6 \times 11 cm) column. The column, of sufficient length to clearly separate a mixture of blue dextran and 32 P, had previously been washed free of metal ions with 10^{-2} M EDTA followed by extensive washing with double quartz-distilled water. Prior to use, the column was equilibrated with Tris-Hepes buffer which had been freed of metal ions by passage through Chelex resin. All of the glassware used had been soaked overnight in 4 N HCl and extensively washed with double quartz-distilled water.

Tests for Lipoate and Cystine—L-Ribulose-5-P 4-epimerase (0.4 μ mole, 85% pure) was incubated at room temperature with 10^{-1} M, 10^{-2} M, and 10^{-4} M Na_2SO_3 in 0.05 M Tris-Hepes buffer, pH 8.0. The activity remaining after 30 min was determined using the continuous assay as described under "Methods." Alternatively, the enzyme was incubated as above with 10^{-2} M mercaptoethanol or 10^{-3} M dithiothreitol. After 30 min, sodium arsenite was added to a concentration of 10^{-1} M to 10^{-4} M. Aliquots of the enzyme were removed after 5 min and the activity determined in the continuous assay.

RESULTS

Metal Ion Activation

Effect of Metal Chelators on L-Ribulose-5-P 4-Epimerase Activity—Although L-ribulose-5-P 4-epimerase does not require added organic or metal cofactors for activity, it is possible that tightly bound metal ions may participate in catalysis. As a first test of this hypothesis, the 4-epimerase was incubated with a series of metal chelators for various incubation times (Fig. 1). A wide variety of responses was observed including both inhibition and stimulation of activity. Complete inhibition was obtained only with 10^{-3} M EDTA. Although 2×10^{-3} M 8-hydroxyquinoline sulfonate did not alter the enzyme activity (not shown), 8×10^{-2} M 8-hydroxyquinoline sulfonate inhibited 90%. 2,3-Dimercapto-1-propanol (10^{-3} M) and mercaptoethanol (10^{-1} M) caused 30 to 60% loss in activity over the 2-hour period (also not shown in Fig. 1).

In contrast, an initial activation was obtained with either dithiothreitol, 1 mM *o*-phenanthroline, or 1 M mercaptoethanol. These results suggest that the enzyme, as isolated, may bind a variety of metals including those species which inhibit. Thus, EDTA may inactivate by removing all metal ions, whereas other metal chelators such as *o*-phenanthroline may activate by preferentially complexing certain inhibitory metal ions. This possibility is supported by the fact that the stability constants for Mn-*o*-phenanthroline or Mn-8-hydroxyquinoline chelates are two or more decades lower than those for heavy metals, whereas the Mn-EDTA stability constant is extremely high and in the same range as those for the heavy metals (12). However, the possibility that the metal chelators may nonspecifically activate or inactivate by means other than removal of a metal ion must be considered.

Activity of L-Ribulose-5-P 4-Epimerase after Removal of EDTA—In order to determine whether inactivation of the L-ribulose-5-P 4-epimerase by EDTA was due to chelation of metal ions or to binding of EDTA to the enzyme, it was necessary to determine the activity of the treated enzyme after removal of the EDTA. For this purpose, the enzyme was inactivated by incubation with 10^{-2} M EDTA for 1 hour at room temperature. The EDTA was then separated from the enzyme by passage through a Sephadex G-25 column as described under "Methods." All buffers used to elute the enzyme from the column and used in the enzyme assay were treated to remove trace contaminations of metal ions as described under "Methods."

The enzyme activity recovered from the Sephadex column varied from 0 to 10% of the initial activity. In the experiment cited in Table I no activity remained. In other cases where low activity remained, it was not ascertained how much was attributable to inaccuracies of the two-step assay, incomplete removal of metal, or recontamination by metal during passage

through Sephadex. At any rate when metal ions were added to the first step of the two-step assay, the activity was greatly increased; the increase depended upon the metal ion species present as detailed below.

Divalent Metal Ion Specificity—To determine the activating capability of various metal ions, the 4-epimerase was dialyzed overnight against 0.05 M Tris-Hepes buffer, pH 8.0, treated with EDTA, freed of EDTA on Sephadex G-25, and assayed in the presence of varying quantities of specific divalent metal ions as described under "Methods." The metal salts used were freshly prepared solutions of spectrographically analyzed metal salts containing less than 5 ppm of most other metals. Under the conditions and concentrations used no precipitation of Mn was observed either in reagents or incubation mixtures. Since, in a preliminary test, the same activity was obtained when the enzyme was previously incubated with 10^{-3} M Co^{++} for 0, 10, or 30 min, the enzyme was not previously incubated with metal ions prior to assaying. Rather, metal ions and substrate were incubated to allow temperature equilibration of the assay mixture, and the reaction was started by the addition of a very small volume of the 4-epimerase.

The results in Table I show that dialysis against Tris-Hepes buffer resulted in an activity loss of about 3-fold, presumably due to loss of metal ion. Following treatment with EDTA and passage through Sephadex G-25 no activity remained. After incubation with metals the highest 4-epimerase activity was obtained with Mn^{++} , and this activation occurred at the lowest divalent cation concentration. A 17-fold stimulation over the activity present in the dialyzed preparation was observed. The MnSO_4 concentration was almost optimal at 10^{-5} M (17-fold versus 18-fold stimulation at 10^{-3} M), whereas 10^{-4} M NiSO_4 and 10^{-3} M or higher MgSO_4 were required for the maximum activation. Similar activating effects were obtained using Cl^- salts of metal ions, thus indicating that a specific anion is not required.

To show the importance of EDTA treatment in obtaining full activation as described above, the 4-epimerase (90% pure) was dialyzed for 2 hours against 0.05 M barbital buffer, pH 8.0, then incubated for 1 hour with 10^{-2} M Co^{++} , Mn^{++} , Zn^{++} , and MgCl_2 salts, and assayed in the two-step assay. Contaminating metals were not removed from the glassware or reagents. The results presented in Table II indicate that only Mn^{++} can stimulate 4-epimerase which had not been treated with EDTA. However, only a 2-fold stimulation was obtained, indicating that Mn^{++} was not able to activate completely without prior EDTA treatment. These results suggest that various nonactivator divalent cations are bound to the Mn^{++} binding site of the 4-epimerase as isolated. These dissociate slowly even in the presence of Mn^{++} , as reported for phosphoglucomutase by Ray (13).

Specific Activity of Crystalline L-Ribulose-5-P 4-Epimerase in Presence of Mn^{++} —Since the preceding results strongly indicated that L-ribulose-5-P 4-epimerase was activated by metal ions, Mn^{++} being the most active, it was necessary to redetermine the specific activity of homogeneous Mn^{++} 4-epimerase.

The L-ribulose-5-P 4-epimerase was twice crystallized as previously reported (1). The second crystals were at least 98% pure as determined by polyacrylamide gel electrophoresis. The enzyme solution was then incubated with 10^{-2} M EDTA, and the EDTA was removed by passage through a Sephadex G-25 column as before. The metal-free enzyme was incubated with 10^{-4} M MnSO_4 (spectro-pure) and assayed with the two-step assay to which 10^{-5} M MnSO_4 had been added. A specific ac-

TABLE I

Divalent metal ion activation of L-ribulose-5-P 4-epimerase

The 4-epimerase (85% pure) was dialyzed overnight against 0.05 M Tris-Hepes buffer, pH 8.0, incubated for 1 hour with 10^{-2} M EDTA, and passed through a Sephadex G-25 column (0.6×11 cm) which had been washed free of cations with EDTA and equilibrated with 0.05 M Tris-Hepes buffer, pH 8.0. Activity was determined in the two-step assay containing spectro-pure metals at the levels indicated in the table. Precautions were taken to remove the contaminating metals from the glassware and the reagents as described under "Methods."

Conditions	Activity units/mg protein	Activation ^a at metal ion concentration of			
		10^{-5} M	10^{-3} M	10^{-4} M	10^{-2} M
Original enzyme	10.0				
After dialysis	3.4				
After EDTA treatment	0.25				
After Sephadex G-25	0.0				
MnSO_4		15	17	17	18
CoSO_4		9.4	11	15	18
NiSO_4		0.65	1.3	6.2	5.3
CaCl_2^b			2.4		2.8
ZnSO_4		0.27	1.1	0.97	1.0
MgSO_4		0.11	0.09	0.09	0.38

^a Expressed as -fold activation over the original activity.

^b CaCl_2 was Mallinckrodt analytical reagent grade.

TABLE II

Effect of divalent metal ions on L-ribulose-5-P 4-epimerase

The L-ribulose-5-P 4-epimerase (specific activity, 10.0) was dialyzed for 2 hours against 0.05 M barbital buffer, pH 8.0, and incubated for 1 hour with 10^{-2} M Co^{++} , Mn^{++} , Zn^{++} , and Mg^{++} as chloride salts and assayed in the two-step assay in the presence of glycylglycine buffer, pH 8.0.

Additions	Original activity
	%
CoCl_2	89
MnCl_2	205
ZnCl_2	13
MgCl_2	78

tivity of 70 ± 7 units per mg of protein was obtained for the pure L-ribulose-5-P 4-epimerase, as compared with 12 units per mg of protein for the crystalline enzyme not so treated (1).

Mechanistic Studies

Effect of Arsenite and Sulfite—Since the 4-epimerase is devoid of NAD^+ , it was considered possible that the epimerization process may involve an oxidation-reduction mechanism using enzyme-bound oxidized lipoic acid or cystine as an electron acceptor. If this were true, either arsenite or sulfite should inhibit the 4-epimerase since dihydrolipoate and cysteine irreversibly react with sulfite.

Accordingly, L-ribulose-5-P 4-epimerase (85% pure) was incubated with 10^{-1} M, 10^{-2} M, 10^{-3} M, and 10^{-4} M sodium sulfite or sodium arsenite with and without prior incubation with either 10^{-2} M mercaptoethanol or 10^{-3} M dithiothreitol to reduce any

disulfide bonds, as described under "Methods." The epimerase was inactivated no more than 10% in any of these experiments, as determined in the continuous assay.

Effect of Sodium Borohydride Treatment—Less than 20% of the epimerase activity was lost on incubation with NaBH_4 in the presence or absence of substrate using the method of Ingram and Wood (14). In addition, when the 4-epimerase was incubated with borohydride in the presence of L-ribulose-5-P and 10^{-4} M CoCl_2 , there was less than a 10% loss in activity. Likewise, the 4-epimerase at pH 6.5 in 0.05 M phosphate buffer was not inactivated by borohydride either in the presence or absence of substrate. The conditions used in these experiments are routinely used by others in this laboratory to obtain complete inactivation of 2-keto-3-deoxy-6-phosphogluconic aldolase in the presence of pyruvate, or of the pyridoxal phosphate-containing L-threonine dehydrase.

Test for Carbanion Intermediate—Carbanions react with tetranitromethane with the liberation of nitroformate which absorbs at 350 nm. Christen and Riordan (15, 16) have used this reagent to demonstrate the presence of a carbanion intermediate in both the yeast (Class II) and the muscle (Class I) fructose diphosphate aldolase-catalyzed reactions. Thus, it is reasonable to assume that if 4-epimerization of L-ribulose-5-P and D-xylulose-5-P were proceeding via a carbanion intermediate, it should be detected by tetranitromethane.

Nitroformate was produced in the presence of either L-ribulose 5-phosphate alone or the 4-epimerase (80% pure) alone in imidazole, glycylglycine, and Tris buffers; pure 4-epimerase did not react with tetranitromethane in Tris buffer. However, the rate of the reaction with L-ribulose-5-P plus the 4-epimerase was not significantly greater than the sum of the individual rates of reaction. In addition, increasing the amount of enzyme did not increase the rate of the nitroformate formation. In a control with FDP aldolase, it was possible to obtain a net increase in 350 nm absorbance with the rate of reaction being dependent upon the aldolase concentration.

DISCUSSION

If L-ribulose-5-P 4-epimerase functions by a mechanism similar to that of UDP-galactose 4-epimerase, there must be a group on the enzyme capable of oxidizing the hydroxyl group on carbon

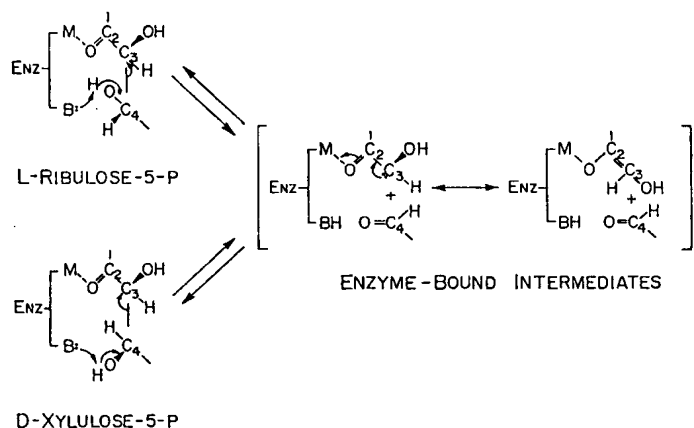


FIG. 2. Proposed dealdolization-aldolization mechanism for L-ribulose-5-P 4-epimerase. *M* depicts a divalent metal ion in the active site and *B*: indicates a base function in the active site.

atom 4 of the substrate. However, previous results have indicated that NAD^+ is not present and is not required for enzyme activity (1). In addition, there were not chromophoric groups as are characteristic of many prosthetic groups and coenzymes. Since the 4-epimerase requires only the addition of metal ions for activity, the putative oxidation-reduction mechanism would have to involve only metal ions and the constituent amino acids.

Cystine and lipoic acid have reduction potentials comparable to that of NAD^+ and, thus, could participate in the epimerization reaction. Presumably, the disulfide form would be required. Since sulfhydryl groups are often readily oxidized by air, the oxidized form could predominate in the active site. Although lipoic acid absorbs at 330 nm, its extinction coefficient is too low to have been readily detected in previous spectral studies (1). However, the evidence discussed below tends to eliminate the SH-disulfide oxidation-reduction mechanism. First, borohydride should reduce the disulfide bond with loss in activity. Concerning the possibility that the disulfide may have been quickly reoxidized prior to or during the assay, it has been observed that activity was not lost on incubation with 1 M mercaptoethanol for 1 hour followed by assay in the presence of 0.05 M mercaptoethanol; that is, under conditions which are usually sufficient to reduce and maintain the integrity of disulfide groups. Although 50% of the activity was lost on incubation with mercaptoethanol for an additional hour, the activity was not recovered on passage through the Sephadex column, suggesting that the activity loss was due to some phenomenon other than reduction of a disulfide bond at the active site. Second, arsenite should have reacted with the reduced disulfide and caused inactivation, and third, sulfite should have reacted with the disulfide group to form the stable sulfur-sulfonated derivative.

No data have been obtained to indicate that an indolenine intermediate derived from tryptophan functions as the electron acceptor in the 4-epimerization as reported by Schellenberg for alcohol dehydrogenase (17).

Consequently, the previous results (1) and those presented herein are not consistent with the electron-acceptor being NAD^+ , cobamide coenzyme, lipoate, cystine, or an oxidized indolenine derivative of tryptophan.

In the absence of any substantial evidence for participation of an oxidizing group on the enzyme, it is necessary to consider other mechanisms for epimerization such as: (a) a Sn_2 (Walden) inversion at C-4; (b) carbon-carbon bond cleavage and re-forma-

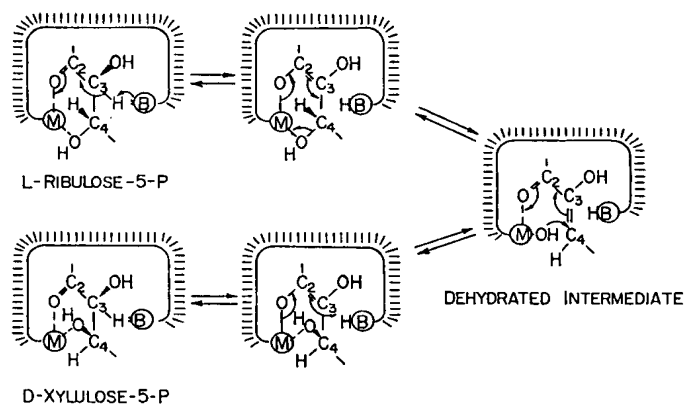


FIG. 3. Proposed dehydration-rehydration mechanism for L-ribulose-5-P 4-epimerase.

tion between C-3 and C-4; and (c) dehydration-rehydration at the same location. A $\text{S}_{\text{N}}2$ inversion is not considered probable since McDonough and Wood (18) previously reported no isotope incorporation into L-ribulose-5-P and D-xylulose-5-P when the epimerization was conducted in H_2^{18}O .

The mechanism proposed in Fig. 2 depicts a metal ion-assisted aldolytic cleavage in a manner strictly analogous to the Schiff base mechanism (19, 20). The metal ion chelates with the carbonyl group (and possibly a hydroxyl group) and serves as an electrophile. A base on the enzyme surface acting as a nucleophile impinges on the C-4 hydroxyl group. In the ensuing rearrangement of electrons, C-3—C-4 cleavage occurs and C-3 takes on carbanion character. In completion of the rearrangement, a metal-oxygen bond is formed at C-2 along with a double bond at C-2—C-3. These intermediates would be analogous to the enamine and ketamine intermediates in the Schiff base mechanism. In this scheme, it is not intended to favor a discrete as opposed to a concerted mechanism.

If the characteristics of the epimerase are such that (a) the carbanion of dihydroxyacetone from carbon atoms 1, 2, and 3 cannot be discharged by a proton as in the case of transaldolase (21, 22) and (b) the glycolaldehyde phosphate moiety does not readily dissociate, it would follow that the carbon-carbon bond would immediately re-form. If there were a high probability that the bond between C-4 and the hydroxyl group would reform *cis* or *trans* in this process, epimerization would be observed. In such a mechanism, L-ribulose 5-phosphate 4-epimerase would, in fact, be a special kind of transaldolase to the extent that the dihydroxyacetone moiety does not dissociate. It would differ in that the other fragment, glycolaldehyde phosphate, is bound and precludes other aldehydes functioning in dihydroxyacetone transfer reactions.

If this hypothesis is correct, the carbanion intermediate would be very short lived because the proximity of glycolaldehyde phosphate would favor condensation. In this connection, the reaction of tetranitromethane in fructose diphosphate aldolase- and transaldolase-catalyzed reactions may be observable because dissociation of glyceraldehyde 3-phosphate allows access to the carbanion intermediates.

An alternative mechanism would be dehydration-rehydration by acid-base catalysis as shown in Fig. 3. The first step would involve a base-catalyzed removal of the proton on C-3 leaving either a carbanion at C-3 or a double bond between C-2 and C-3. The presence of the metal ion in the active site would facilitate removal of the C-4 hydroxyl group in a manner proposed for enolase (23) and aconitase (24). In the reverse reaction, the return of the hydroxyl group would have high probabilities of occupying either bonding position.

Since McDonough and Wood (18) were unable to find an incorporation of T or ^{18}O from the medium into the substrate,

the limitation on this mechanism is that the same proton and hydroxyl groups removed must be involved in the reverse reaction. In this connection, Rose (25) has produced evidence with phosphoglucose isomerase that the intramolecular transfer of a proton can be faster than equilibration with the surrounding medium. Thus, it is conceivable that the proton removed from C-3 becomes bound to the enzyme and is not free to diffuse into the medium. The hydroxyl group would probably be chelated by the metal ion in a position where it would be readily accessible to both sides of C-4 but not to the medium.

Neither dealdolization-aldolization nor dehydration-rehydration can participate in the mechanism of the other carbohydrate 4-epimerases since the substrates of all other 4-epimerases do not possess a free carbonyl group which could participate in the mechanism.

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